

Multi-omics framework prioritizes RNA Methylation Modification Genes in Alzheimer's Disease and Functional Validation of ZFP36 in Regulating Neuroinflammation

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Multi-omics framework prioritizes RNA Methylation Modification Genes in Alzheimer's Disease and Functional Validation of ZFP36 in Regulating Neuroinflammation
Running title: ZFP36 IN ALZHEIMER'S DISEASE

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, in which epitranscriptomic modifications, especially RNA methylation, may exert crucial regulatory effects. This study integrated bulk transcriptomics, single-cell sequencing and machine learning to screen RNA methylation-related genes in AD and explore the functional role of zinc finger protein 36 (ZFP36). We screened RNA methylation-related differentially expressed genes (DEGs) by overlapping AD-associated DEGs with RNA methylation-related genes using the GSE84422 dataset. Six hub genes (C3, ZFP36, S100A9, GADD45A, PHGDH, FBXW7) were screened via PPI centrality analysis, LASSO regression and random forest algorithms and validated in GSE122063. ssGSEA immune infiltration analysis showed ZFP36 had the strongest positive correlation with effector memory CD8⁺ T cell enrichment score. Single-cell RNA-seq of human prefrontal cortex samples revealed high ZFP36 expression in microglia, T cells and astrocytes. Consistent with the bioinformatic findings, in vivo experiments in 9-month-old 5xFAD mice verified that ZFP36 overexpression in the hippocampus improved spatial learning and memory. Cell experiments showed that ZFP36 overexpression alleviated LPS-induced inflammatory responses in microglia. Subsequent virtual screening identified the compound MCULE-4171537037 with moderate binding affinity for ZFP36. These findings associate ZFP36 with AD-related neuroinflammatory features and support further evaluation of its potential as a candidate biomarker and therapeutic target.

Keywords: Alzheimer's disease, RNA methylation, ZFP36, neuroinflammation, diagnostic biomarker.

Introduction

Alzheimer's disease (AD) is the most common form of dementia and causes progressive decline in memory, learning, communication, and daily living ability [1-2]. It substantially impairs quality of life and imposes a considerable burden on families and health-care systems [3-7]. Pathologically, AD is characterized by the aggregation of beta-amyloid (A β) and hyperphosphorylated tau proteins in the brain [8-9]. Although AD has been widely studied, current therapies remain limited. Therefore, it is important to identify new biomarkers and modifiable mechanisms for earlier diagnosis and therapeutic development. Recent findings emphasize the significant impact of epigenetic and epitranscriptomic modifications, particularly RNA methylation, on the pathophysiology of AD.

RNA methylation, including m6A, m5C, m1A, and m7G, can affect RNA splicing, stability, translation, and degradation [10-13]. These processes may influence neuronal function, synaptic plasticity, and inflammatory responses in AD [14-16]. Furthermore, bioinformatic analyses have identified multiple dysregulated RNA methylation-related genes in AD, linking them to distinct molecular subtypes and immune characteristics [17-19]. However, many studies have focused on a single RNA methylation type or a small number of regulators. Few studies have integrated bulk transcriptomic data, immune infiltration analysis, single-cell data, and virtual screening to identify RNA methylation-related genes in AD. This limitation results in an incomplete understanding of key regulatory factors, as most investigations rely primarily on differential expression analysis and functional enrichment at the transcriptomic level, without resolving cell-type-specific expression patterns in brain cells such as microglia or astrocytes. Although altered RNA methylation regulators have been associated with immune features and molecular subtypes in AD, the cell-type-specific and functional relevance of individual candidate genes remains incompletely characterized [20-21]. Moreover, the functional roles of key genes in AD mouse models, particularly their relationships with neuroinflammation and cognitive phenotypes, have not been fully established. Therefore, integrating multi-omics data with functional validation is urgently needed to identify key RNA methylation regulators and elucidate their mechanistic roles in AD progression.

The present study systematically screened differentially expressed genes associated with RNA methylation in AD using transcriptomic datasets, single-cell transcriptomics, and machine learning algorithms, constructing a diagnostic model gene set. Through single-sample set enrichment analysis (ssGSEA) scoring and immune infiltration analysis, we further assessed associations between these genes and the AD immune microenvironment. We further characterized the cellular distribution of ZFP36 in a postmortem prefrontal cortex single-cell dataset. We also evaluated ZFP36 in 5 $\times$ FAD mice to obtain experimental evidence for its functional relevance to AD-related phenotypes. This integrated approach aims to identify critical RNA methylation-related genes, particularly ZFP36, and elucidate

their potential mechanisms in AD pathogenesis via neuroinflammation, offering new theoretical insights for the discovery of diagnostic biomarkers and the development of epitranscriptomic intervention strategies.

1. Materials and Methods

1.1 Data Download

The datasets GSE84422, GSE122063, and GSE291605 were downloaded from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) using GEOquery (Version 2.70.0). GSE84422 and GSE122063 were bulk transcriptomic datasets derived from human brain tissues, whereas GSE291605 was a single-cell transcriptomic dataset derived from human postmortem prefrontal cortex samples. GSE84422 and GSE122063 comprised human brain samples generated on GPL96 and GPL16699, respectively. Dataset GSE84422 includes 328 AD samples and 214 Control samples; dataset GSE122063 includes 56 AD samples and 44 Control samples. All AD and Control samples were included in this study. Dataset characteristics are summarized in Table S1. Datasets GSE84422 and GSE122063 were standardized and processed, including probe annotation and normalization, using the R package limma (Version 3.62.1). GSE291605 (Homo sapiens; postmortem prefrontal cortex; GPL24676) included 20 AD and 2 control samples in the analysis (Table S1)

RNA Methylation Related Genes (RNAMRGs) were collected from the Gene Cards database (<https://www.genecards.org/>) using "5-Methylcytosine", "7-Methylguanosine", "N1-Methyladenosine", and "N6-Methyladenosine" as search keywords. Only "Protein Coding" genes with a "Relevance Score > 2" were retained. Additionally, RNA methylation modification-related gene sets from published literature were obtained from PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) using the same keywords. After merging and removing duplicates, a total of 1684 RNA methylation-related genes were obtained. After intersecting the RNAMRG list with genes detected in both GSE84422 and GSE122063 and removing duplicates, 1244 RNA methylation-related genes were retained for subsequent analysis.

1.2 Alzheimer's Disease-Related Differentially Expressed RNA Methylation Modification-Related Genes

Based on the sample grouping of dataset GSE84422, samples were divided into an AD group and a Control group. Differential expression analysis of genes between the AD group and the Control group was performed using the R package limma (Version 3.62.1). The threshold for Differentially Expressed Genes (DEGs) was set as $|\log FC| > 0.25$ and $p \text{ value} < 0.05$. Among these DEGs, genes with $\log FC > 0.25$ were classified as upregulated and those with $\log FC < -0.25$ as downregulated. Differential expression results were visualized using ggplot2. DEGs were intersected with RNAMRGs to obtain

100 RNAMRDEGs, and the overlap was displayed using a Venn diagram. The expression patterns of RNA
101 methylation-related differentially expressed genes were visualized via a heatmap generated using the R
102 pheatmap package (v1.0.12).

103 1.3 GO and KEGG enrichment analysis.

104 GO enrichment of BP, CC, and MF terms and KEGG pathway enrichment were performed for
105 RNAMRDEGs using clusterProfiler (Version 4.10.0), with $p < 0.05$ and $q < 0.25$ as significance
106 thresholds; the five terms with the lowest p values in each category were selected for visualization.

107 1.4 Protein–protein interaction network analysis.

108 A protein–protein interaction (PPI) network of RNAMRDEGs was constructed using the STRING
109 database with a minimum required interaction score > 0.15 . Interacting RNAMRDEGs were ranked in
110 CentiServer using Degree, Betweenness, Closeness, Maximum Neighborhood Component (MNC),
111 Edge Percolated Component (EPC), and Degree Maximum Neighborhood Component (DMNC); the
112 top 10 genes from each algorithm were intersected, and the shared genes were defined as Key Genes.

113 1.5 Machine Learning

114 To obtain model genes for AD, two machine learning algorithms were used: LASSO (Least
115 Absolute Shrinkage and Selection Operator) regression and RF (Random Forest). LASSO regression
116 analysis was performed on the Key Genes using the R package glmnet with parameters `set.seed(2024)`
117 and `family = "binomial"`. The results of LASSO regression were visualized using a diagnostic model
118 plot and a variable trajectory plot. Random Forest analysis was performed using the randomForest
119 package based on the expression levels of the Key Genes in GSE84422, with `set.seed(2024)` and `ntree`
120 `= 1000`. A 5-time 10-fold cross-validation procedure was then performed to determine the optimal
121 number of variables. Important genes were selected according to the cross-validation error and
122 MeanDecreaseGini values for subsequent analysis.

123 The genes shared by LASSO and random forest were defined as Model Genes and visualized using
124 a Venn diagram. Finally, to explore the expression differences of the model genes between the AD and
125 the Control in datasets GSE84422 and GSE122063, grouping comparison plots were generated based
126 on the expression levels of the Model Genes.

127 1.6 Immune Infiltration Analysis (ssGSEA)

128 Single-sample gene set enrichment analysis (ssGSEA) was used to estimate relative enrichment
129 scores for immune cell signatures. Based on previously reported immune cell marker gene sets, ssGSEA
130 was used to estimate the relative infiltration abundance of 28 immune cell types, including Activated

CD8 T cells, Activated dendritic cells, Gamma delta T cells, Natural killer cells, Regulatory T cells, and other human immune cell subtypes. The resulting ssGSEA enrichment scores were compared between AD and control samples in GSE84422 and visualized using ggplot2 (Version 3.4.4). Spearman correlations among immune-cell signatures and between Model Genes and immune-cell signatures were visualized using pheatmap (Version 1.0.12) and ggplot2 (Version 3.4.4), respectively.

1.7 Construction of High and Low RNA Methylation Modification Score Groups

The RNA Methylation Score (RNAM.Score) for all samples was calculated using the R package GSVA with the ssGSEA algorithm, based on the expression matrix of Model Genes in dataset GSE84422. Additionally, a grouping comparison plot was generated using the R package ggplot2 to evaluate the diagnostic performance of the RNAM.Score for AD occurrence. Based on the median value of the RNA Methylation Score (RNAM.Score), AD samples were divided into a high score and a low score. Model Gene expression was compared between the HighScore and LowScore AD groups. The R package pROC was used to generate ROC curves and calculate AUC values. The ROC curve of RNAM.Score was used to evaluate its ability to distinguish AD samples from Control samples in GSE84422. In AD samples, ROC curves of the Model Genes were generated to assess their discriminatory ability between the high- and low-RNAM.Score groups.

1.8 Gene set variation analysis.

GSVA was performed on GSE84422 using the GSVA package (Version 1.50.0) and the c2.cp.v2023.2.Hs.symbols gene set from MSigDB to compare the high- and low-RNAM.Score groups; pathways with $p < 0.05$ were retained, and the top 10 positive and negative pathways ranked by logFC were visualized.

1.9 Single-Cell Dataset Quality Control and Cell Type Annotation.

The Counts matrix of 20 AD samples and 2 Control samples from the Alzheimer's Disease single-cell dataset GSE291605 was imported using the "CreateSeuratObject" function from the R package Seurat (Version 5.0.1) to create a Seurat object. Parameters were set such that genes were expressed in at least 10 cells and each cell had at least 200 genes expressed. Cells with RNA count < 500 , RNA feature count < 250 , $\log_{10}\text{GenesPerUMI} < 0.8$, and mitochondrial gene content $> 20\%$ were filtered out, as low-quality cells or empty droplets typically have fewer genes. GSE291605 was normalized using NormalizeData with the default 'LogNormalize' method, the top 4,000 highly variable genes were selected using FindVariableFeatures with the 'vst' method, and the data were scaled using ScaleData. Principal component analysis (PCA) was performed, and the ElbowPlot function was used to help determine the number of principal components for downstream dimensionality reduction and clustering.

Thirty principal components were retained for downstream UMAP analysis. The "FindNeighbors" function was used with default parameters and the first 10 significant principal components as dimensions to construct K-nearest neighbors based on Euclidean distances in the PCA space. Cells were clustered into different clusters using the "FindClusters" function, with a resolution of 0.6 determined by the "clustree" function. Finally, the "RunUMAP" function was used for dimensionality reduction to visualize and explore the dataset.

Cell type annotation for the AD single-cell dataset GSE291605 was performed using the "SingleR" function from the R package SingleR (Version 2.4.1), based on the reference dataset Human Primary Cell Atlas Data and cell marker genes. Cell-type proportions were summarized using bar plots; UMAP was used to visualize cell clusters, and DotPlot and UMAP were used to display marker-gene and Model Gene expression across cell types and clusters.

1.10 Virtual Screening (DrugCLIP)

To identify candidate compounds with potential binding affinity to the Model Genes, DrugCLIP was used for virtual screening of the corresponding proteins. Protein structures obtained from PDB were preprocessed, and CB-Dock2 was used to identify binding pockets; the largest C1 pocket was selected as the target region, its center coordinates and radius were recorded, and the processed structures and pocket parameters were then submitted to DrugCLIP for large-scale virtual screening. The output results were sorted in descending order by DrugCLIP score, with the top-ranked compounds considered candidate molecules. A Vina score > -5 kcal/mol indicates very weak or no binding; scores between -7 and -5 kcal/mol indicate moderate binding affinity; and scores < -7 kcal/mol indicate strong binding affinity.

1.11 Mice

All mice were maintained on a C57BL/6 background. WT mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, whereas 5×FAD mice were obtained from Nanjing Biomedical Research Institute. Animals were bred and kept in an SPF facility at Hubei University of medicine Center for Animal Experiment. All animal experiments were done with approval by the Institutional Animal Care and Use Committee of Hubei University of medicine and in accordance with institutional regulations.

1.12 Animal Model and Stereotactic Injection

Three-month-old female 5×FAD mice and age-matched wild-type (WT) littermates were used in this study. For ZFP36 overexpression or knock down, 5×FAD mice received bilateral hippocampal stereotactic injection of AAV9-TTP OE or AAV9-TTP KD (1.5×10^{13} vg/mL, 300 nL per side).

Stereotactic coordinates relative to bregma were set as: anteroposterior -2.0 mm, mediolateral ± 1.5 mm, dorsoventral 1.5 mm. Empty AAV9 vector was injected as the control. Three months after viral injection, Y-maze assays were performed as previously described. At nine months of age, mice were euthanized by excessive CO₂ inhalation. For tissue preparation, mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde to fix hippocampal tissues for subsequent histological and molecular analysis.

1.13 Behavioral test

Y-maze assays were conducted to evaluate short-term working memory and long-term spatial reference memory in mice. All behavioral tests were performed in a quiet, uniformly lit laboratory. Mice were habituated to the testing environment for 1 h before each trial to reduce stress interference. All operations were completed by a blinded experimenter to avoid subjective bias. The maze was disinfected with 75% ethanol after each test to eliminate olfactory cues and guarantee experimental stability.

The spontaneous alternation test was performed to evaluate working memory. Mice were individually placed at the end of a random Y-maze arm facing the center and allowed to explore freely for 5 min. A valid entry was defined as full entry of all four paws into one arm. Total arm entries and sequential tri-arm alternations were recorded by behavioral analysis software. The alternation rate was calculated as follows: $\text{Alternation (\%)} = (\text{Number of successful alternations}) / (\text{Total arm entries} - 2) \times 100$.

The novel arm recognition test was performed 24 h later to assess spatial reference memory. During training, one arm was blocked as the novel arm, and mice explored the remaining two arms for 5 min. After a 1-h recovery interval in their home cages, the novel arm was unblocked, and mice were allowed to explore the entire maze for 5 min. Arm entry frequency and exploration duration were recorded to analyze spatial memory preference.

The Morris water maze (MWM) test was applied to evaluate spatial learning and memory in mice. On the first experimental day, a visible platform was placed above the water surface. The escape latency and swimming speed of each mouse were recorded to screen for qualified mice with normal vision, motor ability, and exploratory motivation. Starting from the second day, a hidden platform was adopted for continuous positioning and navigation training for five consecutive days, with escape latency recorded daily. On day 7, the platform was removed, and a 60 s spatial probe test was performed. The number of platform crossings and the residence time in the target quadrant were analyzed to assess spatial memory retention.

1.14 Immunohistochemical Staining

Hippocampal tissue sections were placed in antigen retrieval solution and continuously heated at low temperature for 1 minute. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 15 minutes. After blocking with 1% BSA, sections were incubated with Iba1 antibody (RRID: AB_3711469) overnight at 4°C. HRP-conjugated goat anti-rabbit IgG (RRID: AB_228341, 31460, ThermoFisher, USA) was incubated for 1 hour at 37°C. Staining was performed using DAB and hematoxylin. Sections were dehydrated in ethanol, cleared in xylene, and mounted with neutral gum. Quantification and analysis of immunohistochemical staining were performed using Image-Pro Plus version 6.0 software.

1.15 Western Blotting

Total protein was extracted using RIPA lysis buffer, and its concentration was quantified using a BCA kit. Polyacrylamide gels consisted of 5% stacking gel and 8%, 10%, and 12% separating gels. 20 µL of sample (30 µg protein) was subjected to SDS-PAGE to separate target proteins. Proteins were transferred to PVDF membranes, which were then blocked with blocking solution (SW3010, Solarbio, China) for 1 hour. After incubation with primary antibodies overnight at 4°C, PVDF membranes were incubated with secondary antibodies. Antibody incubation was performed for 1 hour at 37°C. Finally, protein bands were visualized using ECL chemiluminescent substrate.

1.16 Real time PCR

First-strand cDNA was synthesized from 1 µg of total RNA using the HiScript II Q RT SuperMix (Vazyme, China). Quantitative real-time PCR (qRT-PCR) was performed on a BIO-RAD CFX 96 TOUCH (BIO-RAD, USA). The amplification reaction was prepared using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China), according to the standard protocols. The PCR thermal cycling conditions were set as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s. The relative mRNA expression levels of target genes were calculated via the delta-delta-Ct method and normalized to GAPDH. All primer sequences used in this study are listed in table1.

Target	Forward	Reverse
Human TTP	TCGGGACCCTGGAGCCTGAG	AGCCAGCGGTGCGAAGCC
Mouse TTP	CCATCTACGAGAGCCTCCAGTC	AGTCCGAGTTTATGTTCCAAAGTCC
Human IL-6	AGTGAGGAACAAGCCAGAGC	GGTCAGGGGTGGTTATTGCA
Mouse PHGDH	CCTCATTGTCCGGTCTGCTAC	CATCTTTCATCGAAGCTGTTGC

FBXW7	GCCTGAGCATGTCCACGTTA	ACTTCAGAACCATGGTCCAACCTT
S100A9	ATGGTGGAAGCACAGTTG	TGGTTTGTGTCCAGGTCC
C3	TGCTGGCCTCTGGAGTAGAT	AGGCAGTCTTCTTCGGTGTG
GADD45A	AGACCGAAAGGATGGACACG	GCAGGCACAGTACCACGTTA
Human IL-1 β	AGTGGCAATGAGGATGACTTGT	TGTAGTGGTGGTCGGAGATTC
Human TNF- α	CTTCCAGCTGGAGAAGGGTG	CCCAAAGTAGACCTGCCCAG
Human GAPDH	ACCATCTTCCAGGAGCGAGATC	TGATGACCCTTTTGGCTCCCC
Mouse GAPDH	CTATTGGCAACGAGCGGTTCC	GCACTGTGTTGGCATAGAGGTC

Table 1 The sequence of primer

1.17 Cell Culture and Treatment

Human microglial cell line HMC3 was purchased from Procell system and cultured in MEM supplemented with 10% fetal bovine serum at 37 °C under 5% CO₂. To establish an LPS-induced inflammatory activation model, HMC3 cells were stimulated with 10 μ M LPS for 24 h for subsequent functional assays. For TTP gain- and loss-of-function experiments, cells were transfected with LV-TTP OE or LV-TTP KD for 48 h prior to LPS stimulation.

1.18 Total RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood by density gradient centrifugation. Total RNA was extracted from PBMCs with TRIzol reagent. RNA concentration and purity were determined by NanoDrop 2000 spectrophotometry. All participants provided written informed consent prior to peripheral blood sample collection. All experimental procedures complied with the Committee on the Ethics of human Experiments of Hubei University of medicine.

1.19 Antibodies and other reagents

Antibodies for A β , TTP (ZFP36) were provided by Abcam and Millipore company respectively. Other antibodies used in this study include: β -actin (Proteintech Group) Goat Anti-Rabbit IgG-HRP (Proteintech Group), Goat Anti-Mouse IgG-HRP (Proteintech Group). PAMPs used in this study were LPS (Escherichia coli 0111: B4, L2630, Sigma)

1.20 Statistical Analysis

All analyses were performed in R (Version 4.3.3), with continuous variables summarized as mean $\pm$ standard deviation. Two-group and ≥ 3 -group comparisons used the Wilcoxon rank-sum and Kruskal–Wallis tests, respectively; categorical variables were compared using the chi-square or Fisher’s exact test, Spearman correlation was used unless otherwise specified, and $p < 0.05$ was considered statistically significant.

2. Results

2.1 Screening of Differentially Expressed RNA Methylation Modification-Related Genes (RNAMRDEGs) and Functional Analysis of RNAMRDEGs in Alzheimer's Disease

GSE84422 was used for discovery, GSE122063 for expression validation, and GSE291605 for single-cell analysis. Compared with controls, 119 genes were differentially expressed in AD samples ($|\log FC| > 0.25$, $p < 0.05$), including 35 upregulated and 84 downregulated genes (Fig. 2A). Intersection with the 1,244 RNAMRGs yielded 18 RNAMRDEGs (Fig. 2B-C). These results suggest that these RNAMRDEGs, including ZFP36 and GADD45A, may be associated with AD-related molecular changes.

To further explore functional network of 18 differentially expressed genes in AD, GO and KEGG enrichment analyses were performed for the 18 RNAMRDEGs (Table S2). GO analysis highlighted terms related to the p38MAPK cascade, inflammatory responses, Toll-like receptor binding, and the CCR4-NOT complex, with the complete enrichment results provided in Table S2 and Fig. 2D. KEGG analysis identified enrichment in pathways annotated as thyroid cancer, endometrial cancer, basal cell carcinoma, melanoma, and non-small cell lung cancer (Fig. 2D). Network plots for BP, CC, MF, and KEGG pathways were also generated based on the enrichment analysis, suggesting that ZFP36 and GADD45A/B were associated with p38MAPK cascade in biological processes. ZFP36 was involved in CCR4-NOT complex, S100A8, S100A9, and C3 mapped to the secretory granule lumen and cytoplasmic vesicle lumen terms. S100A8 and S100A9 were key genes in Molecular Functions. GADD45A and GADD45B were the central genes in KEGG pathways (Fig. 2E-H). Network plots including the Toll-like receptor binding, p38MAPK cascade, tumor associated pathways were significantly enriched. These enrichment results suggest that the RNAMRDEGs are associated with inflammatory and signaling pathways relevant to AD.

2.2 Network Regulation Analysis of RNAMRDEGs and Model Gene Construction

To analyze the protein-protein interactions of these 18 RNAMRDEGs, The PPI Network analysis indicated that 15 of the 18 RNAMRDEGs had interaction relationships (Fig. 3A). Through the

comprehensive evaluation of the six algorithms, 6 key genes were ultimately identified in all algorithms, including FBXW7, PHGDH, ZFP36, C3, S100A9, and GADD45A (Fig. 3B).

To further prioritize candidate genes, LASSO regression and random forest analyses were applied. In the LASSO model, the minimum lambda value was used as the optimal solution based on the Key Genes. Both LASSO and random forest retained the same six candidate genes (Fig. 3C-G). The overlap between genes selected by the two algorithms was displayed using a Venn diagram. The results showed that these 6 genes-C3, ZFP36, S100A9, GADD45A, PHGDH, and FBXW7-are core genes in Alzheimer's Disease (Fig. 3H). Both LASSO and random forest selected FBXW7, PHGDH, ZFP36, C3, S100A9, and GADD45A (Fig. 3H). The consistency between LASSO regression and Random Forest supports the robustness of these six genes as candidate model genes for subsequent AD-related analyses.

To confirm the expression of these 6 Model Genes (C3, ZFP36, S100A9, GADD45A, PHGDH, FBXW7) in Alzheimer's Disease patients in datasets GSE84422 and GSE122063, expression analysis was performed. In dataset GSE84422, C3 and PHGDH were significantly up-regulated, while ZFP36, FBXW7, S100A9, and GADD45A were significantly down-regulated (Fig.4A). In dataset GSE122063, C3, ZFP36, PHGDH, and S100A9 were significantly up-regulated, FBXW7 was down-regulated, and GADD45A showed no statistically significant difference (Fig.4B). Overall, most Model Genes showed significant expression differences between AD and Control samples in the validation dataset GSE122063, whereas GADD45A did not reach statistical significance in this dataset. These findings showed that several candidate genes remained differentially expressed in GSE122063, although the magnitude and direction of the associations varied across genes and datasets.

2.3 Immune Infiltration Analysis (ssGSEA) in Alzheimer's Disease Patients

To characterize immune-related associations of the Model Genes, ssGSEA was used to estimate enrichment scores for 28 immune cell signatures. The correlations between Model Genes and these immune cells were also analyzed to investigate the immune microenvironment characteristics of AD and the potential immunoregulatory functions of the Model Genes. Fourteen immune-cell signatures showed differential enrichment in GSE84422 (Fig. 4C). Among immune-cell signatures, effector memory CD8⁺ T cells and natural killer cells showed the strongest positive correlation ($r = 0.64$, $p < 0.05$; Fig. 4D). ZFP36 showed the strongest positive association with the effector memory CD8⁺ T cell signature ($r = 0.763$, $p < 0.05$), whereas FBXW7 was negatively correlated with the central memory CD4 T-cell signature ($r = -0.464$, $p < 0.05$; Fig. 4E). These results suggest that the Model Genes are associated with specific immune cell infiltration patterns in AD and may be involved in AD-related immune microenvironment changes.

2.4 Construction of the Group of High and Low RNA Methylation Modification Score and Gene Set Variation Analysis (GSVA)

RNAM.Score was significantly lower in AD than in control samples ($p < 0.001$; Fig. 5A). Grouping comparison plots illustrated the expression differences of the 6 Model Genes between these two groups in AD samples. The expression levels of S100A9, ZFP36, GADD45A and C3 were markedly decreased in the high-score subgroup (Fig. 5B). ROC analysis showed that C3, PHGDH, and FBXW7 had low discriminatory accuracy for distinguishing HighScore and LowScore groups ($0.5 < \text{AUC} < 0.7$), whereas ZFP36 and GADD45A showed moderate discriminatory accuracy ($0.7 < \text{AUC} < 0.9$), and S100A9 showed high discriminatory accuracy ($\text{AUC} > 0.9$) (Fig. 5C–H, Table.S3).

GSVA analysis revealed the low score was mainly enriched in immune and inflammatory response pathways related to metal chelation, complement cascade, and macrophage marker pathways. In contrast, the high Score was enriched in metabolic and genetic information processing pathways such as mitochondrial RNA degradation and asparagine metabolism (Fig. 5I–J). These GSVA findings support an association between the expression-based RNAM.Score and immune-inflammatory pathway activity in AD.

2.5 Single-Cell Dataset Analysis of Model Gene Expression

Given the significant correlations between Model Genes ZFP36, GADD45A, C3, etc., and immune-inflammatory responses in AD (Figs 2-5), We next analyzed GSE291605 to characterize the cell-type-specific expression of the six Model Genes (Fig. 6A). Eighteen distinct cell clusters were identified in an integrated dataset consisting of 62,287 cells (Fig. 6B). These clusters were further classified into eight cell populations based on cell markers, namely astrocytes, endothelial cells, excitatory neurons, inhibitory neurons, microglia, oligodendrocytes, oligodendrocyte precursor cells (OPCs), and T cells (Fig. 6C). The expression levels of 24 marker genes across the single-cell dataset verified the reliability of marker gene expression and cell clustering (Fig. 6D). There were differences in cell proportions among different groups (Fig. 6E). We also analyzed the cell proportions difference between the AD and normal. Compared with the Control group, the AD group showed a lower proportion of oligodendrocytes and a higher proportion of astrocytes (Fig. 6F–G), suggesting alterations in brain cell composition in AD.

Subsequently, the expression of six model genes across different groups were displayed in the single-cell dataset. The results demonstrated that C3 was highly expressed primarily in microglia, oligodendrocytes, T cells and inhibitory neurons under AD conditions. ZFP36 exhibited prominent high expression in microglia, T cells and astrocytes. FBXW7 was broadly upregulated across all eight cell

populations (Fig. 7A-F). ZFP36 therefore showed a cell-type-specific expression pattern involving microglia, T cells, and astrocytes.

2.6 Virtual Screening of Lead Compounds Targeting Model Genes via DrugCLIP

To identify candidate compounds with potential binding affinity to the Model Genes C3, FBXW7, GADD45A, PHGDH, S100A9, and ZFP36, we performed virtual screening of these proteins using the DrugCLIP tool. DrugCLIP identified the top 100 candidate molecules with putative high binding affinity for each target protein (Table S4), which were ranked from high to low according to DrugCLIP scores (Table S4).

Moderate binding affinity was observed between C3 and STK424251 (Vina score = -6.42 kcal/mol), FBXW7 and STK617630 (Vina score = -6.69 kcal/mol), GADD45A and Z15671386 (Vina score = -6.79 kcal/mol) (Fig. 8A-C), S100A9 and Z115632122 (Vina score = -6.57 kcal/mol), as well as ZFP36 and MCULE-4171537037 (Vina score = -6.66 kcal/mol) (Fig. 8D-F). Strong binding affinity was detected between PHGDH and Z8781413865 (Vina score = -8.13 kcal/mol). MCULE-4171537037 possesses great binding potential toward ZFP36. These results provide theoretical clues for further validation of candidate compounds targeting AD-related Model Genes.

2.7 The elevated expression of ZFP36 in AD.

To further confirm the expression patterns of the six hub genes in AD pathogenesis, a 9-month-old symptomatic 5×FAD mouse model was established to detect their mRNA expression levels (Fig. 9A–B). Compared with control mice, the mRNA expression of ZFP36 and C3 was significantly upregulated in AD mice. However, the other model genes showed no significant changes (Fig. 9G). Notably, ZFP36 exhibited consistent and significant upregulation in vivo AD models. Therefore, ZFP36 was selected as the core target for subsequent functional exploration.

Next, hippocampal, frontal cortical, and cerebral cortical tissues were isolated from mice to detect the mRNA and protein expression of ZFP36. Western blot results showed that ZFP36 protein levels were markedly increased in the hippocampus, frontal cortex, and cerebral cortex of 5×FAD mice (Fig. 9C). qRT-PCR results were consistent with the protein changes (Fig. 9D). For clinical validation, peripheral blood samples were collected from 6 AD patients and 6 healthy normal controls. The results indicated that ZFP36 mRNA expression was significantly upregulated in AD patients compared with normal individuals (Fig. 9E). Furthermore, bioinformatics analysis based on multiple NCBI public databases further verified the increased ZFP36 expression in brain tissues of AD patients (Fig. 9F). Collectively, these cumulative findings strongly suggest the potential regulatory role of ZFP36 in the progression of AD.

2.8 Overexpression of ZFP36 improves cognitive impairment in 5xFAD mouse model

To investigate the function of ZFP36 in AD, effects of hippocampal ZFP36 overexpression were subsequently evaluated in 5xFAD mice (Fig. 10A). In the Y mazes pace exploration experiment, the ZFP36-OE group mice crossed the original platform position significantly more times than the control group, and the time they stayed in the target quadrant was also significantly prolonged (Fig. 10B). Spontaneous alternations and time involved arm visits are much more than the 5xFAD mice (Fig. 10C-D). These results indicate that overexpression of ZFP36 in the hippocampus can significantly improve the spatial learning and memory abilities of 5xFAD mice.

2.9 Overexpression of ZFP36 markedly alleviated LPS-triggered microglial inflammation.

Prior to formal experiments, the optimal LPS concentration (Fig. 11A-C), as well as the transfection efficiency of lentivirus-mediated TTP overexpression and knockdown (Fig. 11D), was determined. qPCR analysis demonstrated that TNF- α and IL-6 mRNA levels were reduced in ZFP36-overexpressing HMC3 cells following LPS stimulation (Fig. 11E-F). The data demonstrates a protective upregulation of ZFP36 occurs in AD, and elevated ZFP36 expression substantially mitigates neuroinflammation in AD pathology.

3. Discussion

Our integrated analyses prioritized six RNA methylation-related Model Genes-C3, ZFP36, S100A9, GADD45A, PHGDH, and FBXW7 and highlighted ZFP36 for further investigation in relation to immune and inflammatory phenotypes. ZFP36 is an RNA-binding protein with established roles in limiting inflammatory signaling through the destabilization of AU-rich element-containing transcripts [22]. ZFP36 can target and bind to AU-rich elements (AREs) located in the 3'UTR of pro-inflammatory factor mRNAs, triggering their degradation and thereby modulating disease progression [23-25]. ZFP36 showed close associations with immune infiltration patterns and was mainly expressed in microglia, T cells, and astrocytes in the single-cell dataset. These findings prioritize ZFP36 as a candidate identified from an RNA methylation-related gene set and link its expression to immune and inflammatory features of AD. Through model gene screening and validation analysis, ZFP36 showed potential value as a candidate RNA methylation-related biomarker in AD. However, whether ZFP36 participates in the early stages of AD progression requires further longitudinal and experimental validation.

Single-cell analysis showed prominent ZFP36 expression in microglia, T cells, and astrocytes, suggesting that these cell populations may be relevant to its role in AD-associated neuroimmune responses. Single-cell analysis also showed differences in cellular composition between AD and control samples, including a higher astrocyte proportion and a lower oligodendrocyte proportion in AD.

Together with the expression of ZFP36 in microglia and astrocytes, these observations support further investigation of ZFP36 within glial inflammatory contexts, although the limited number of control samples precludes firm conclusions about cell-composition changes. Notably, the positive correlation between ZFP36 expression and effector memory CD8⁺ T cell abundance suggests that ZFP36 may be associated with T-cell-related immune features in AD, but the functional direction of this association requires further validation. As an RNA-binding protein that promotes degradation of inflammatory cytokine mRNAs via AU-rich elements, ZFP36 upregulation in AD could serve as a critical hub for modulating immune-inflammatory pathways [22]. This is supported by studies showing it suppresses inflammation by destabilizing target mRNAs, such as those encoding VEGF-C and mTORC2 axis components [24], and is associated with regulation of cellular senescence and ferroptosis [25]. A growing body of evidence highlights the critical role of RNA modifications in regulating neuroinflammatory processes driving AD [26-27]. The lower RNAM.Score observed in AD samples was accompanied by enrichment of complement-, macrophage-, and other immune-inflammatory signatures in the LowScore group. This pattern suggests that the expression profile captured by RNAM.Score is associated with an inflammatory transcriptional state, although it should not be interpreted as evidence of reduced RNA methylation itself. However, because RNAM.Score was calculated from gene expression profiles rather than direct RNA methylation measurements, this relationship should be interpreted as an expression-based association. In APP/PS1 mice, elevated m6A abundance in the hippocampus has been associated with microglial signatures, including phagocytosis and inflammatory signaling, suggesting that RNA methylation-related changes may influence microglial function.

Our findings also point to dysregulation of specific RNA modification readers and target genes. Previous studies have reported that disruption of RNA modification machinery may affect post-transcriptional regulation of genes involved in neuroinflammation, supporting the biological relevance of RNA modification-related genes in AD [28]. For instance, the long non-coding RNA SNHG1, regulated by transcription factor GATA4 through a methylation-involving pathway, promotes A β -induced neuroinflammation and cognitive impairment [29-32]. Differential expression of genes like C3, S100A9, and GADD45A in our study is supported by literature linking them to neuroinflammation: Among the other candidate genes, C3 has been implicated in complement-mediated synaptic and inflammatory changes in AD [33-34], whereas S100A9 has been linked to inflammatory and amyloidogenic responses in the central nervous system [35-36]. The dysregulation of genes such as C3, S100A9, and GADD45A may reflect RNA methylation-related transcriptional changes and may be associated with inflammatory processes involving microglia and astrocytes in AD.

In 5 $\times$ FAD mice, ZFP36 protein and mRNA levels were increased in several AD-related brain regions, including the hippocampus, frontal cortex, and cerebral cortex. These findings provide

experimental support for an association between ZFP36 expression and AD-related brain phenotypes. ZFP36 overexpression in 5×FAD mice improved spatial learning and memory, supporting a potential protective role for ZFP36 in AD. The mechanism by which ZFP36 overexpression ameliorated cognitive impairment in 5×FAD mice were recapitulated in HMC3 cell-based experiment, we found that ZFP36 overexpression reduced TNF- α and IL-6 expression after LPS stimulation in HMC3 cells. Although the LPS model is not AD-specific, these findings provide independent cellular support for an anti-inflammatory effect of ZFP36. This aligns with previous findings that ZFP36 overexpression in APP/PS1 mice ameliorated cognitive deficits and suppressed NLRP3 inflammasome activation via ZBP1 mRNA degradation [22]. Virtual screening identified the lead compound MCULE-4171537037, which exhibited moderate binding affinity to ZFP36, providing a preliminary basis for targeted pharmacological interventions.

Despite these findings, several limitations should be acknowledged. First, this study mainly relied on public transcriptomic datasets, and differences in platforms, sample processing, and batch effects may influence the results. Second, the single-cell dataset contained only two control samples, which may reduce the robustness of cell-type-specific comparisons. Third, the immune infiltration and gene-immune cell association analyses were based on computational inference and correlation analysis, so they cannot establish causal regulatory relationships. Fourth, the diagnostic value of the Model Genes requires further validation in larger independent clinical cohorts. Finally, DrugCLIP and docking results remain computational predictions; direct ZFP36 binding, target engagement, and biological activity of the prioritized compounds require experimental validation. Additionally, the functional interaction between astrocytes and ZFP36 was not thoroughly explored, although recent evidence suggests ZFP36 family members are significantly expressed in astrocytes and other glial cells in AD [37-38]. Additional mechanistic studies are needed to define how ZFP36 acts in specific neural and immune cell populations, while larger independent human cohorts are required to assess the generalizability of the transcriptomic findings.

4. Conclusion

In summary, this study prioritized six RNA methylation-related candidate Model Genes-C3, ZFP36, S100A9, GADD45A, PHGDH, and FBXW7-through integrated transcriptomic analysis, PPI analysis, and machine learning. Among them, ZFP36 was closely associated with immune infiltration features and showed prominent expression in microglia, T cells, and astrocytes in the single-cell dataset. DrugCLIP-based virtual screening further identified MCULE-4171537037 as a candidate compound with predicted binding affinity to ZFP36. Furthermore, Overexpression of ZFP36 markedly alleviated microglial inflammation in AD. Together, these findings support further investigation of ZFP36 in AD-

associated neuroinflammation, while its underlying mechanisms and translational relevance require additional validation.

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COMPETING INTERESTS

Author Contributions: DYH, and PPX conceived and designed the experiments. DYH, YHZ and JK performed the experiments. ZHX, and KBZ performed the animal experiments. ZYT and YXYL analyzed the data. RL performed the bioinformatics and statistical analysis of the genomic data. DYH, YHZ and PPX and drafted the manuscript with the input from all authors. All authors discussed the results and approved the final manuscript.

Competing interests: The authors declare no conflicts of interest.

Ethics declarations

Approval for animal experiments: All experimental procedures complied with the Committee on the Ethics of Animal Experiments of Hubei University of medicine (China).

Approval for human experiments: Informed consent was obtained from all participants and/or their legal guardians prior to enrollment in the study.

Data Availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

616

617 FIGURE LEGENDS

618 **Figure 1. Flow Chart for the Comprehensive Analysis**

619 DEGs,Differentially Expressed Genes;RNAMRGs,RNA Methylation Related Genes;RNAMRDEGs,RNA Methylation
620 Related Differentially Expressed Genes;PPI,Protein-protein Interaction;GSEA,Gene Set Enrichment
621 Analysis;GSVA,Gene Set Variation Analysis;GO,Gene Ontology;KEGG,Kyoto Encyclopedia of Genes and
622 Genomes;UMAP,Uniform Manifold Approximation and Projection.

623 **Figure 2. Screening of Differentially Expressed RNA Methylation Modification-Related Genes** 624 **(RNAMRDEGs) and Functional Analysis of RNAMRDEGs in Alzheimer's Disease.**

625 A. Volcano plot of differentially expressed genes analysis between Alzheimer's disease (AD) group and Control group
626 in dataset GSE84422. B. Venn diagram of differentially expressed genes (DEGs) and RNA methylation modification
627 related genes (RNAMRGs) in dataset GSE84422. C. Heat map of RNA methylation-modified differentially expressed
628 genes (RNAMRDEGs) in dataset GSE84422. D. Bubble diagram of gene ontology (GO) and pathway (KEGG)
629 enrichment analysis results of RNA methylation-related differentially expressed genes: biological process (BP), cellular
630 component (CC), molecular function (MF) and biological pathway (KEGG). GO terms and KEGG terms are shown on
631 the abscissa. E-H. Gene ontology (GO) and pathway (KEGG) enrichment analysis results of RNA methylation-related
632 differentially expressed genes network diagram showing BP (E), CC (F), MF (G) and KEGG (H). Pathway enrichment
633 analysis was performed using the KEGG database. KEGG pathway maps are referenced from Kanehisa Laboratories
634 (<https://www.kegg.jp/kegg/kegg1.html>).The screening criteria for gene ontology (GO) and pathway (KEGG) enrichment
635 analysis were p value < 0.05 and FDR value (q value) < 0.25 .

636 **Figure 3. Network Regulation Analysis of RNAMRDEGs and Model Gene Construction.**

637 A. Protein-protein interaction Network (PPI Network) of RNA methylation-related differentially expressed genes
638 calculated from STRING database. B. Upset map of Top10RNA methylation-related differentially expressed genes
639 calculated by six algorithms in CentiServer package. C-D. Diagnostic model plot (C) and variable trajectory plot (D) of
640 LASSO regression model. E. Model training error plot of random forest algorithm. F. MeanDecreaseGini scatter plot of
641 Model Genes (in descending MeanDecreaseGini order). G. Cross validation error plot. H. Intersection Venn diagram of
642 genes screened by two machine learning algorithms.

643 **Figure 4. Immune Infiltration Analysis (ssGSEA) in Alzheimer's Disease Patients.**

644 A-B. Group comparison of Model Genes in the Alzheimer's disease (AD) group and Control group of datasets GSE84422
645 (A) and GSE122063 (B). C. Grouping comparison diagram of immune cells in dataset GSE84422. D. Correlation
646 heatmap of immune cells in dataset GSE84422. E. Bubble plot of correlation between immune cell infiltration abundance
647 and Model Genes in dataset GSE84422. ns represents p value ≥ 0.05 , no statistical significance; * represents p value $<$
648 0.05 , statistically significant; ** represents p value < 0.01 , highly statistically significant; *** represents p value < 0.001
649 and highly statistically significant. The absolute value of correlation coefficient (r value) below 0.3 was weak or no

correlation, between 0.3 and 0.5 was weak correlation, and between 0.5 and 0.8 was moderate correlation. Red is positive correlation, blue is negative correlation. The depth of the color represents the strength of the correlation.

Figure 5. Construction of the Group of High and Low RNA Methylation Modification Score and Gene Set Variation Analysis (GSVA).

A. Comparison of RNA methylation modification Score (NPM.score) between the AD and Control in dataset GSE84422. B. Group comparison diagram of Model Genes between the group of high score and low score of AD samples. C-H. ROC curves of Model Genes S100A9 (C), ZFP36 (D), GADD45A (E), C3 (F), PHGDH (G), FBXW7 (H) in AD. I-J. Heat map (I) and bidirectional bar graph (J) of gene set variation analysis (GSVA) results between high score and low score of dataset GSE84422. ns stands for p value ≥ 0.05 , not statistically significant; * represents p value < 0.05 , statistically significant; *** represents p value < 0.001 , highly statistically significant. When AUC > 0.5 , it indicates that the expression of the molecule is a trend to promote the occurrence of the event, and the closer the AUC is to 1, the better the diagnostic effect. AUC between 0.5 and 0.7 had low accuracy, AUC between 0.7 and 0.9 had moderate accuracy, and AUC above 0.9 had high accuracy. The screening criteria for gene set variation analysis (GSVA) were p value < 0.05 and logFC ranked Top10 positive enrichment pathways and Top10 negative enrichment pathways.

Figure 6. Single-Cell Dataset Analysis of AD patients.

A. Violin plot of gene expression from the Alzheimer's disease (AD) single-cell dataset GSE291605. B. All cells were clustered into 18 cell clusters by UMAP. C. Annotate cells into 8 cell types via the R package singleR and cell marker genes: Astrocyte, Endothelial cells, Excitatory neurons, Inhibitory neurons, Microglia, Oligodendrocyte, OPCs, T cells. D. Bubble plot visualization of expression levels of cell marker genes in eight cell types. E. Bar graph of the proportion of cells in different samples. F. Bar graphs of cell proportions in different groups. G. Visualization of the expression levels of the six Model Genes in bubble plots.

Figure 7. Single-Cell Dataset Analysis of Model Gene Expression.

A-F. UMAP shows the expression levels of Model Genes (C3 (A), ZFP36 (B), S100A9 (C), GADD45A (D), PHGDH (E), FBXW7 (F)) in single cell dataset GSE291605.

Figure 8. Virtual Screening of Lead Compounds Targeting ZFP36 via DrugCLIP.

A-C. Visualization of docking results between C3 and STK424251 (A), FBXW7 and STK617630 (B), GADD45A and Z15671386 (C), from left to right, the global docking map and the interaction force map, respectively. D-F. Visualization of docking results between PHGDH and Z8781413865 (D), S100A9 and Z115632122 (E), and ZFP36 and MCULE-4171537037 (F), respectively, from left to right are global docking maps and interaction force maps.

Figure 9. The elevated expression of ZFP36 in AD.

A. A β staining in 5 $\times$ FAD mice. B. Learning and memory performance scores of 5 $\times$ FAD mice assessed via the Morris water maze test. C. Western blot analysis of ZFP36 protein expression in 5 $\times$ FAD mice. D. Quantitative detection of ZFP36 mRNA levels in 5 $\times$ FAD mice. E. Analysis of ZFP36 expression in peripheral blood of AD patients. F. Analysis

of ZFP36 expression in patients with Alzheimer's disease based on the GEO database. G. Quantitative detection of C3、GADD45A、GHDGH、S100A9、FBXW7 mRNA levels in 5×FAD mice hippocampus.

Figure 10. Overexpression of ZFP36 improves cognitive impairment in 5×FAD mouse model

A. Time schematic diagram of TTP-OE AAV9 virus injection and behavioral experiments in 5× FAD mice. B. Trajectory diagram of each group of mice in the Y-maze test. C. The number of spontaneous alternations was quantified for each group of mice in the Y-maze test. D. Time spent in the target quadrant in the Y-maze test.

Figure 11. Overexpression of ZFP36 markedly alleviated LPS-triggered microglial inflammation.

A-C. The mRNA expression level of pro-inflammatory cytokines following stimulation with different concentrations of LPS. D. Efficiency of lentivirus-mediated ZFP36 knockdown and overexpression in HMC3 cells. E-F. TTP overexpression suppressed the LPS-induced upregulation of pro-inflammatory cytokine mRNAs.

Figure 1.

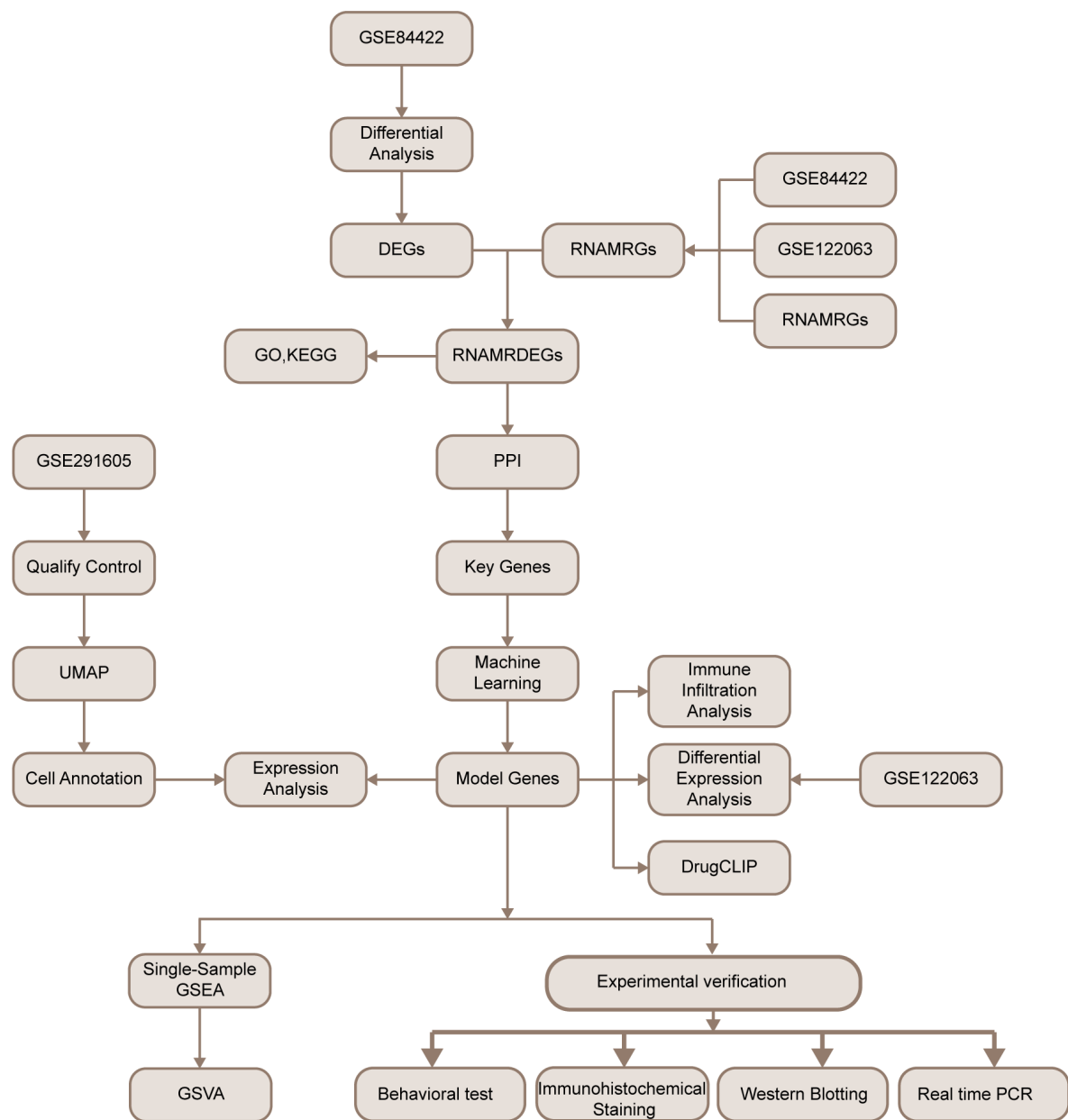
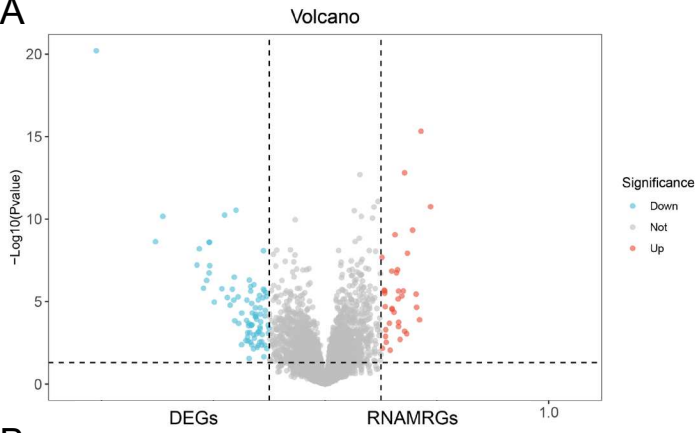
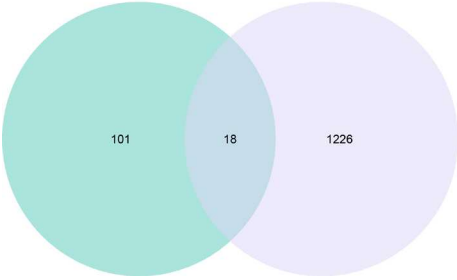


Figure 2.

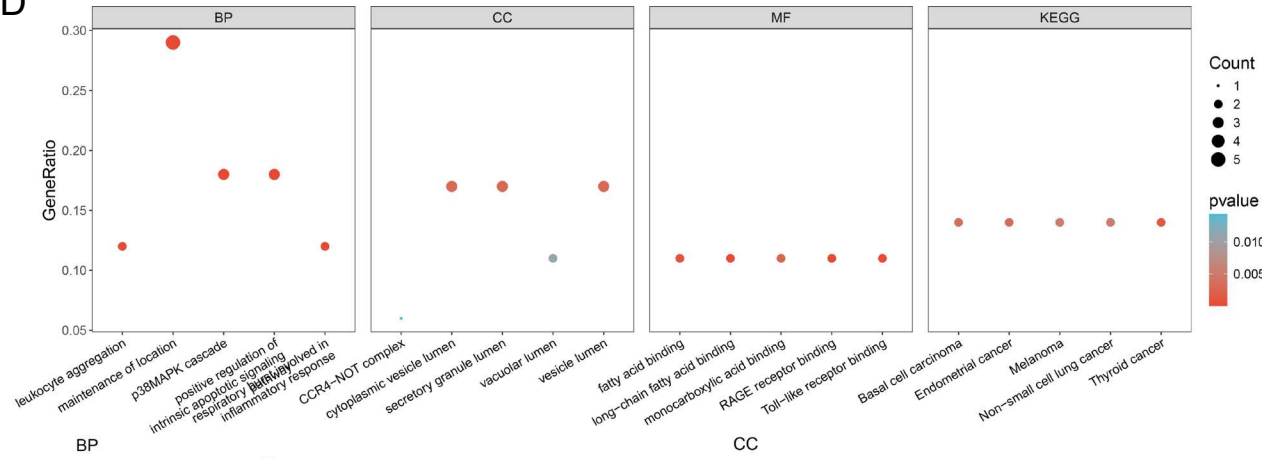
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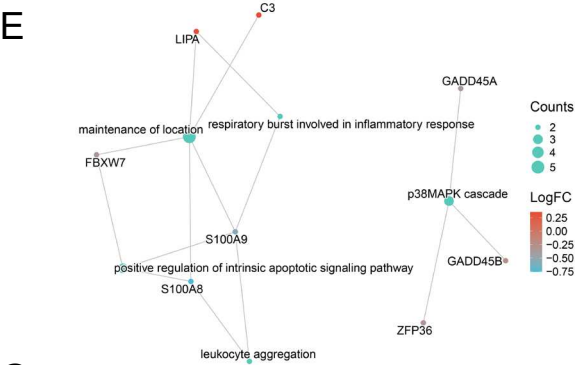
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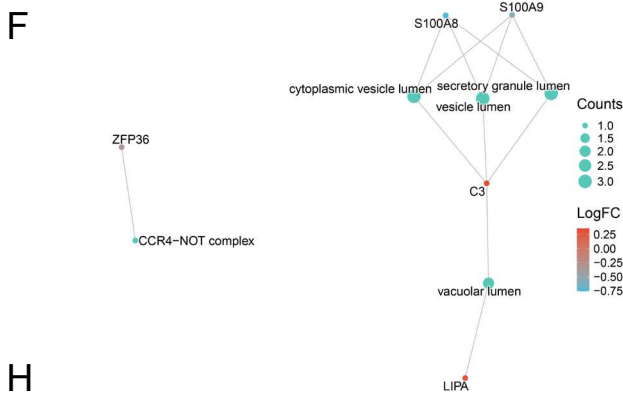
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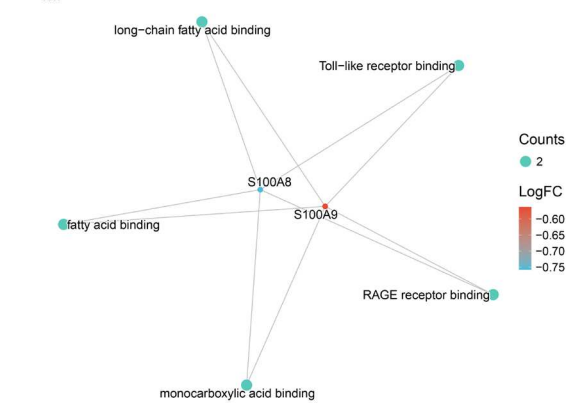
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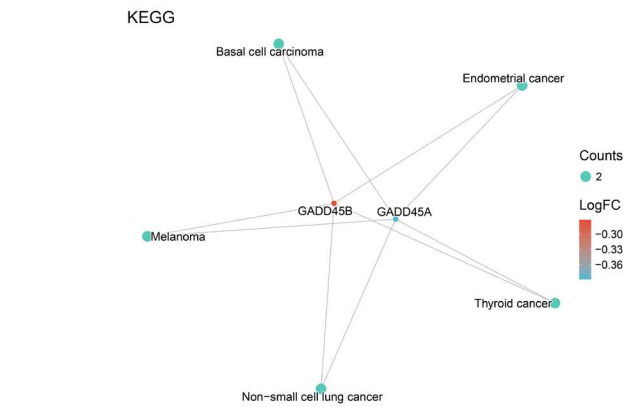
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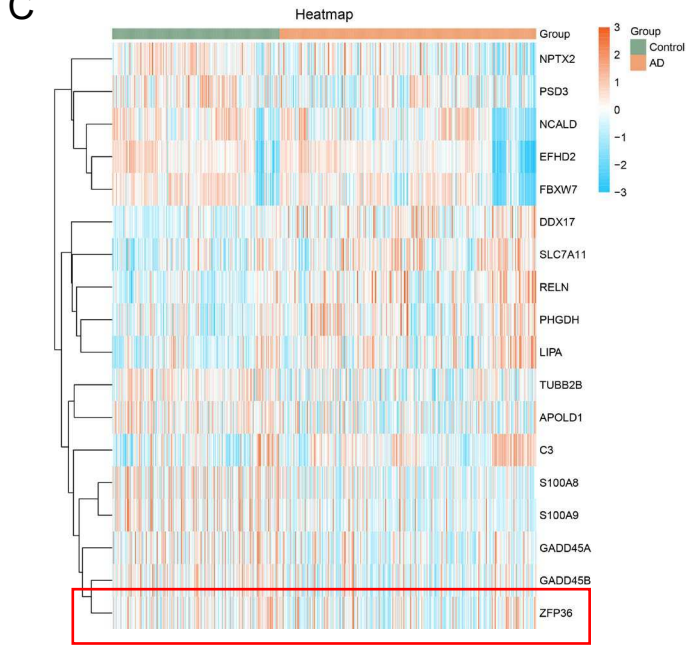


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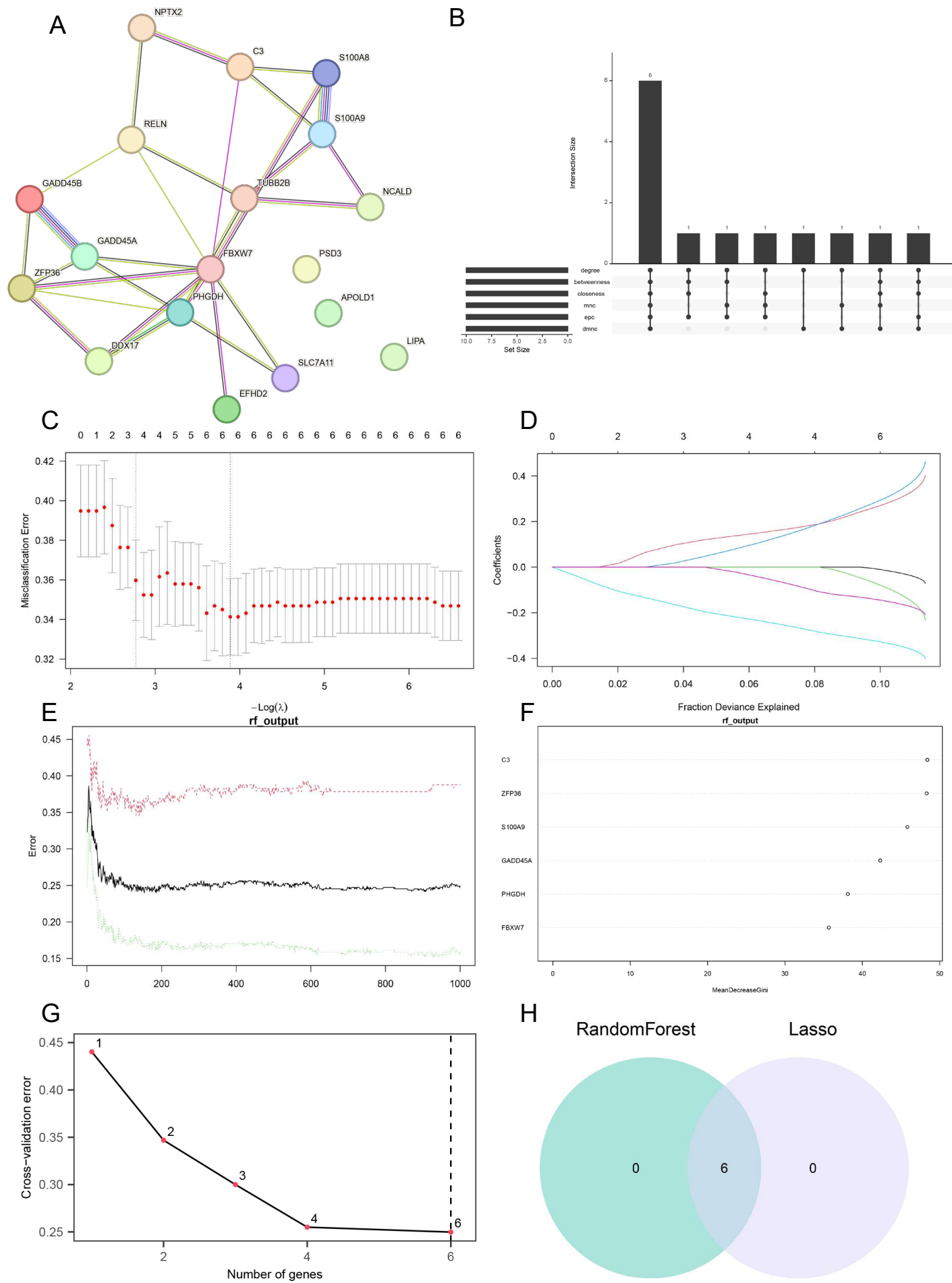


Figure 4.

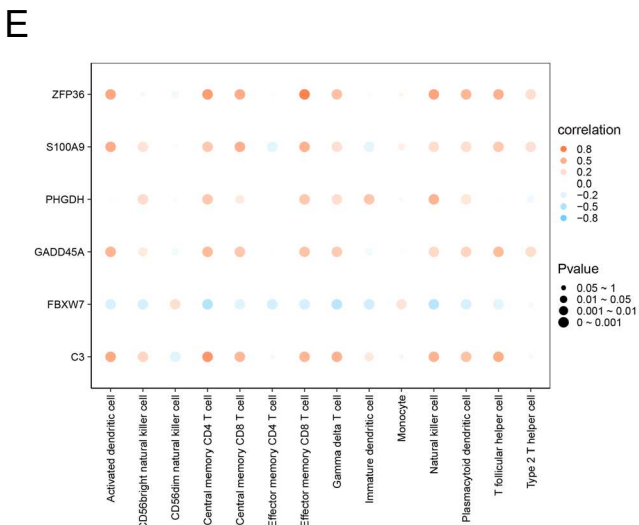
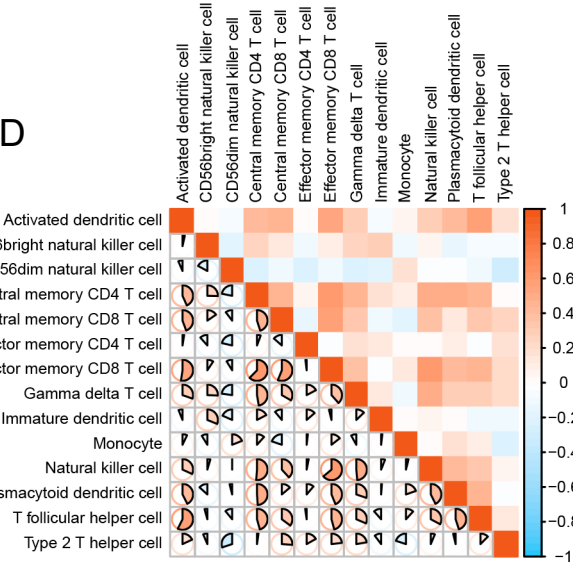
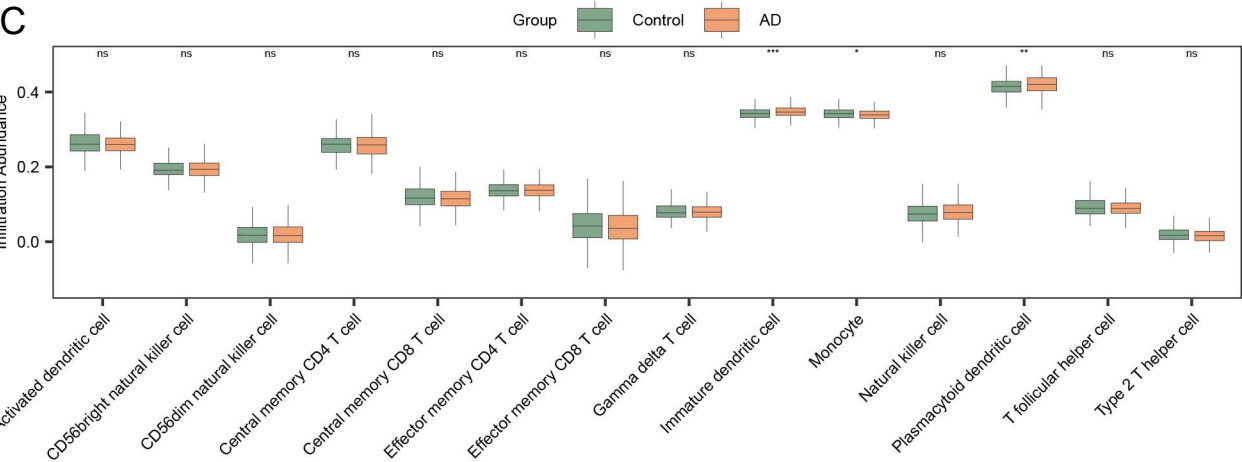
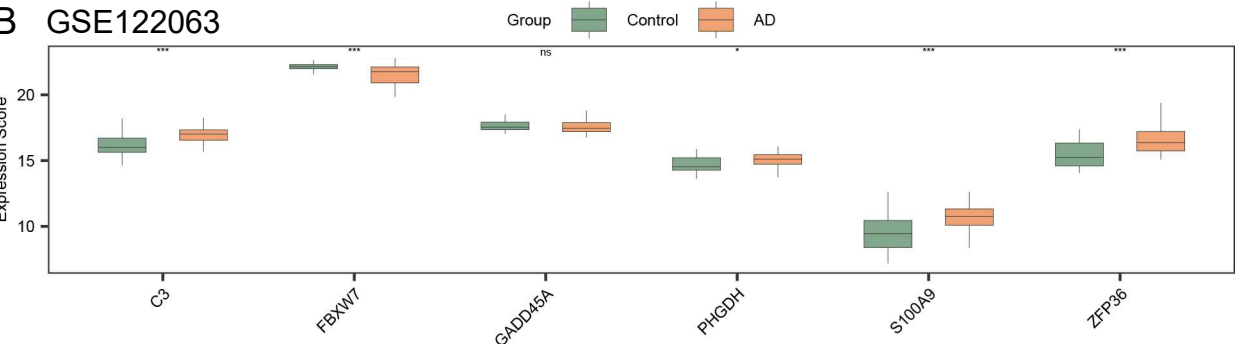
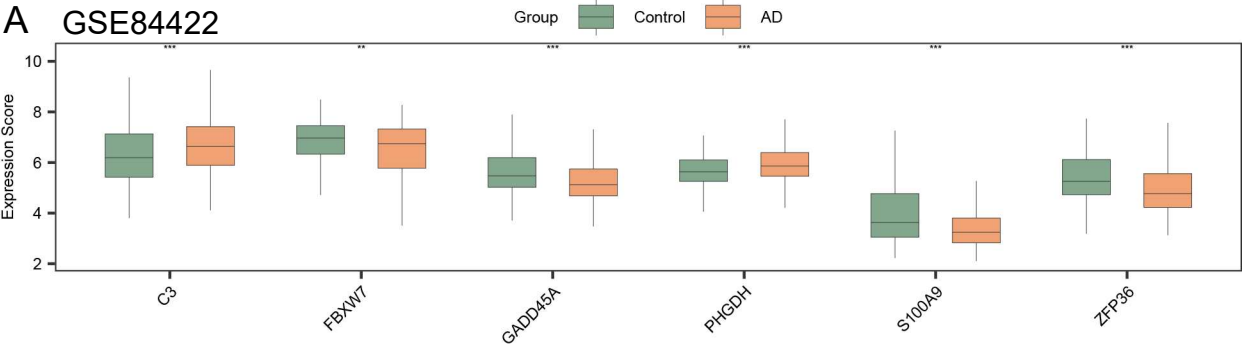


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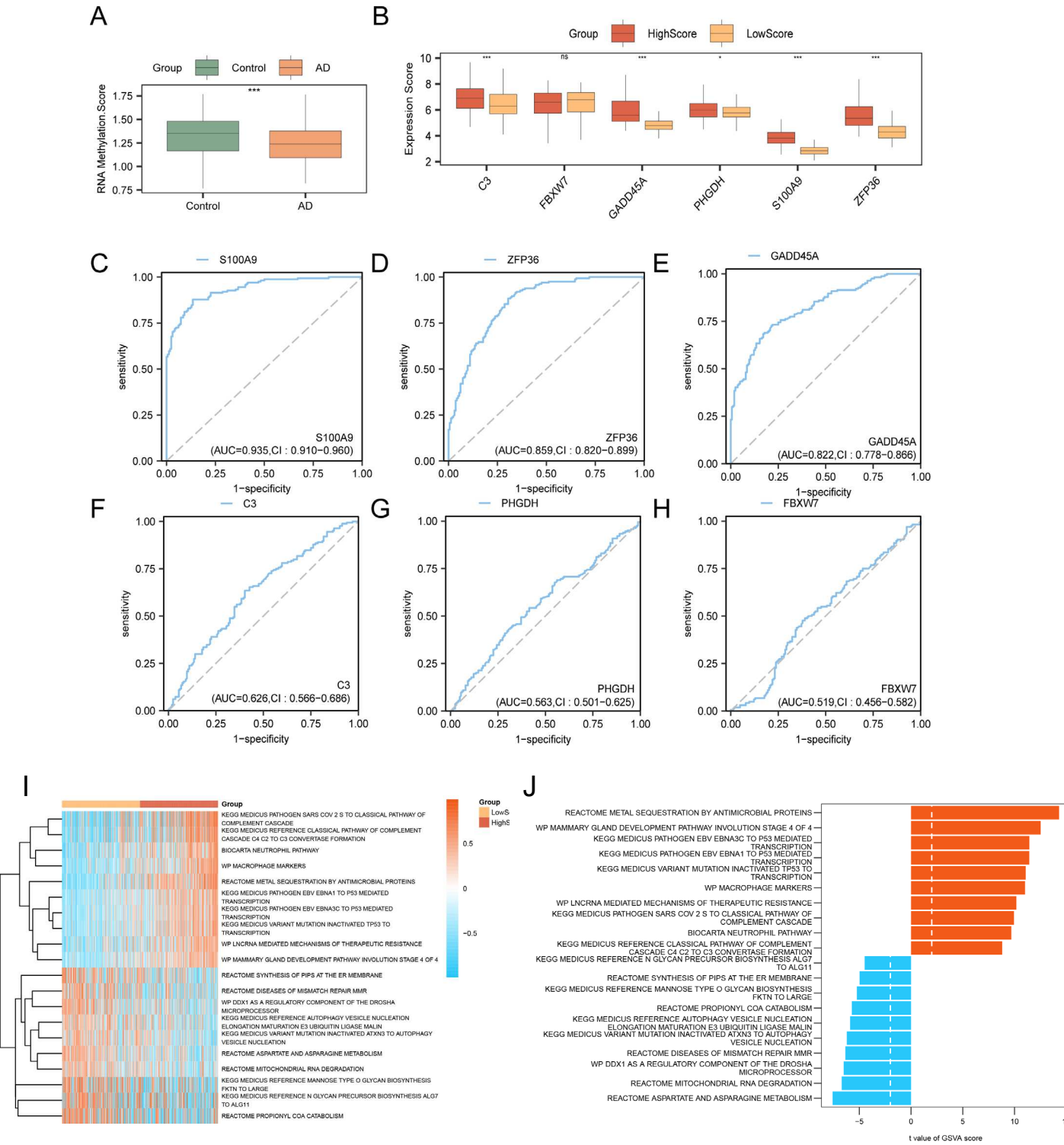


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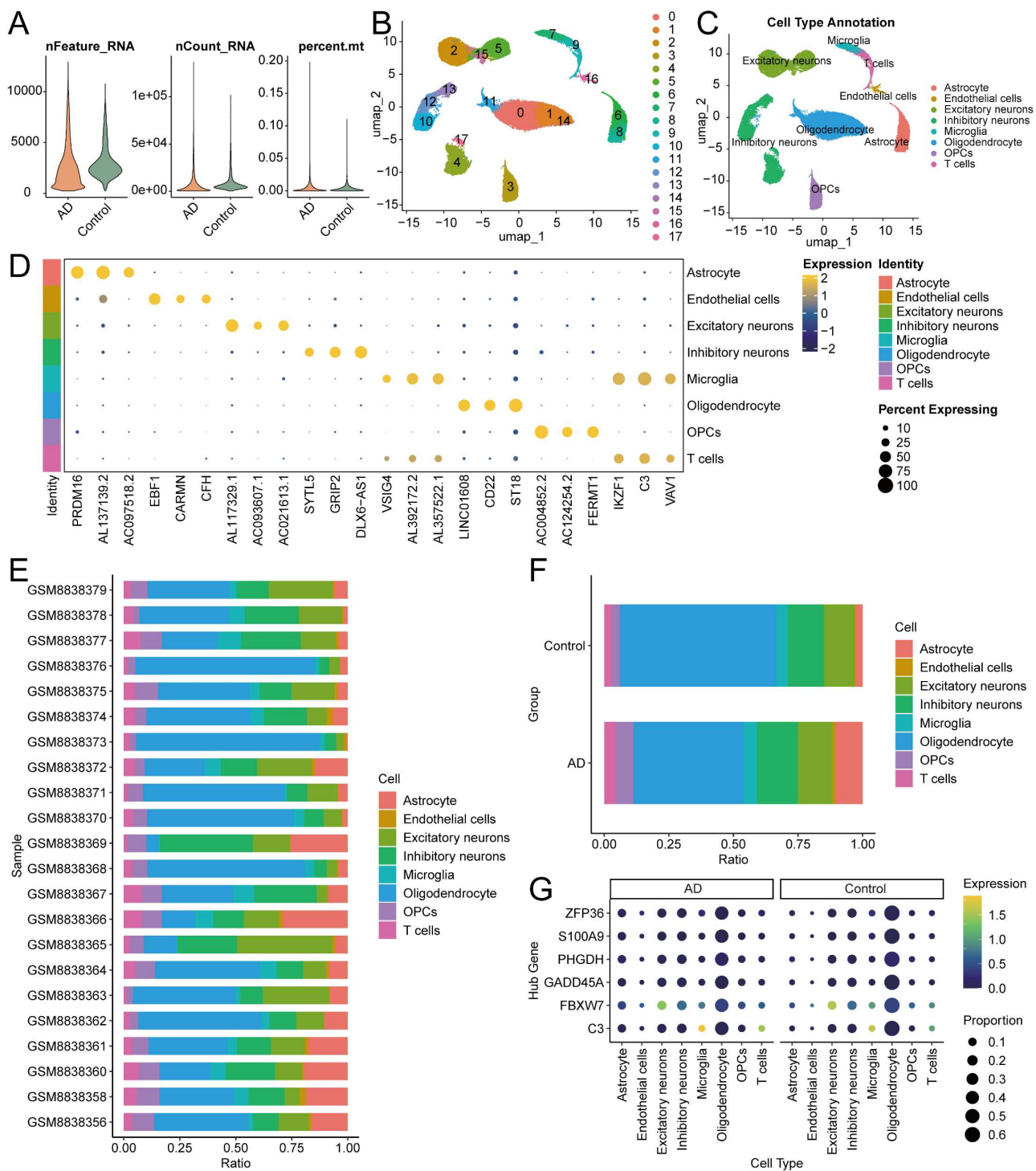


Figure 7.

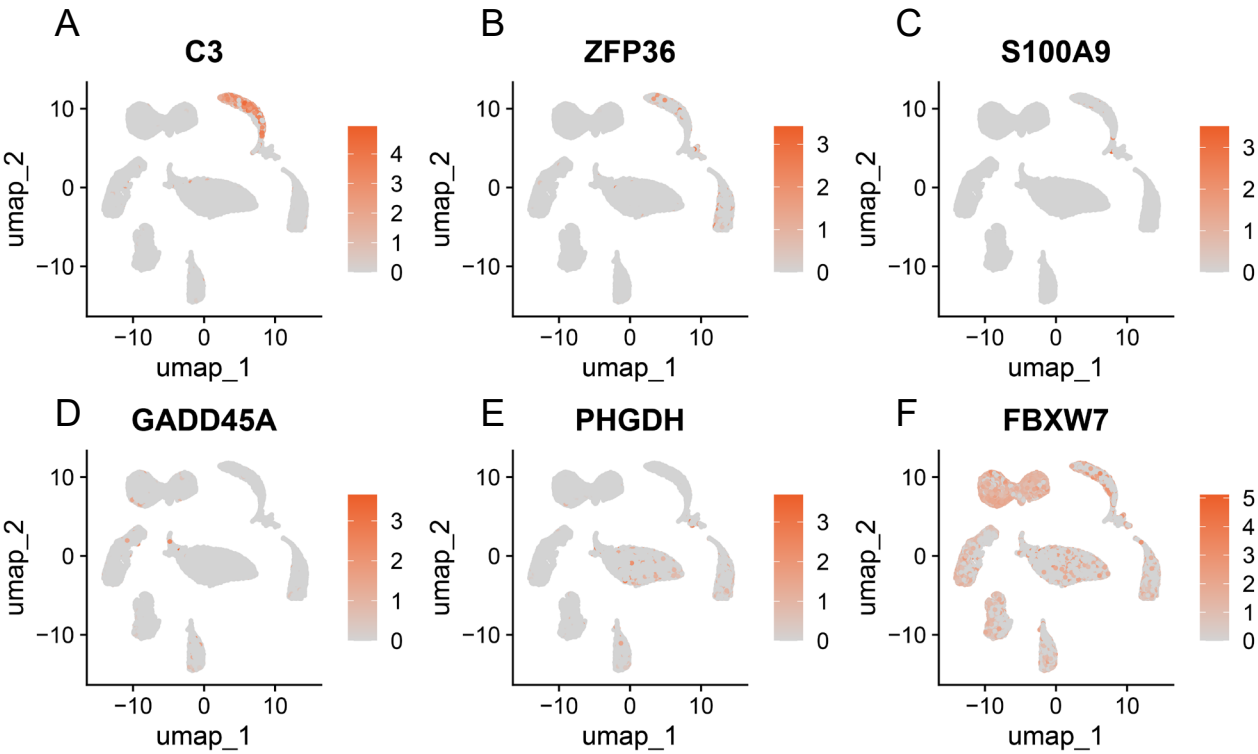


Figure 8.

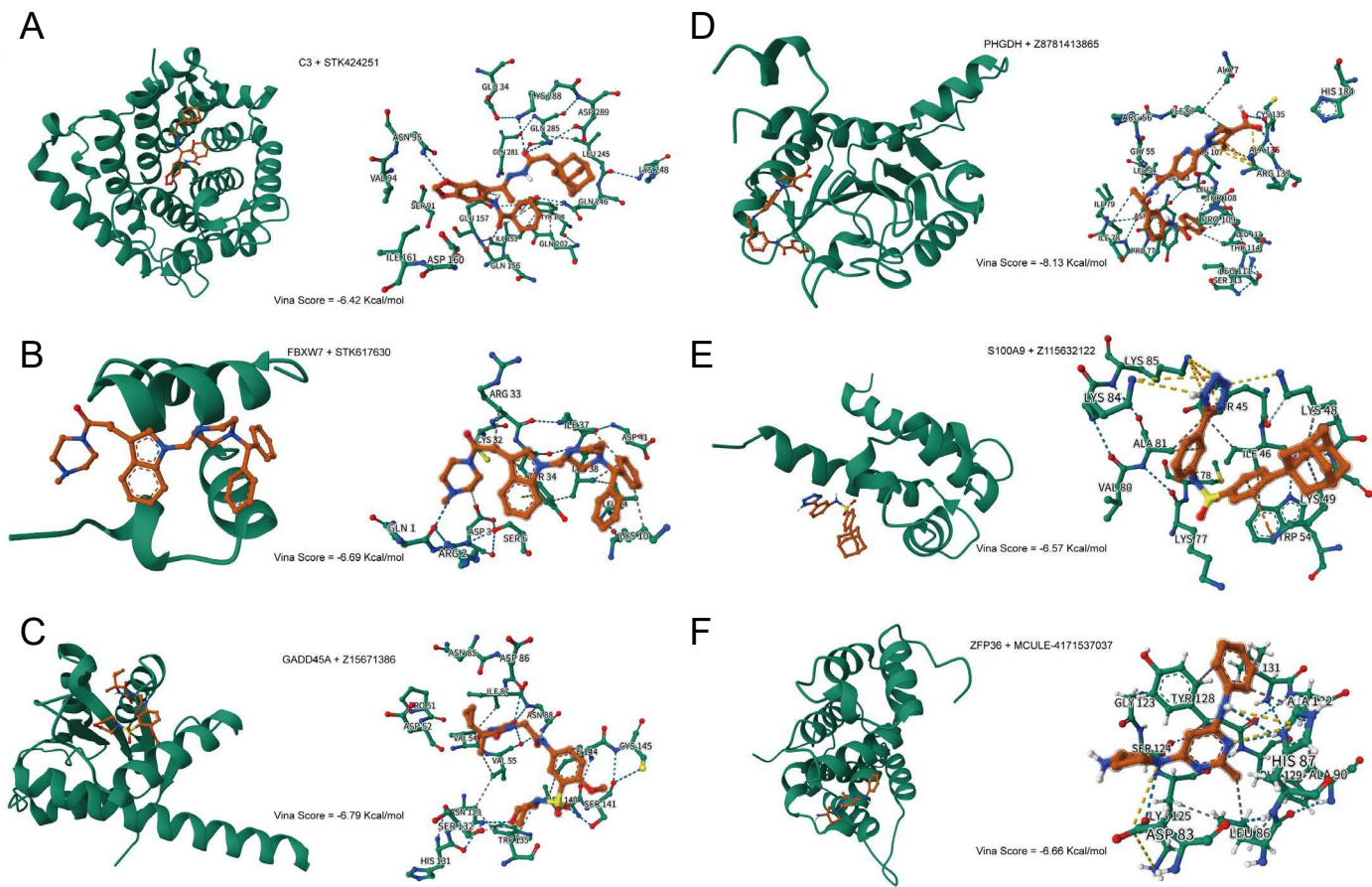


Figure 9.

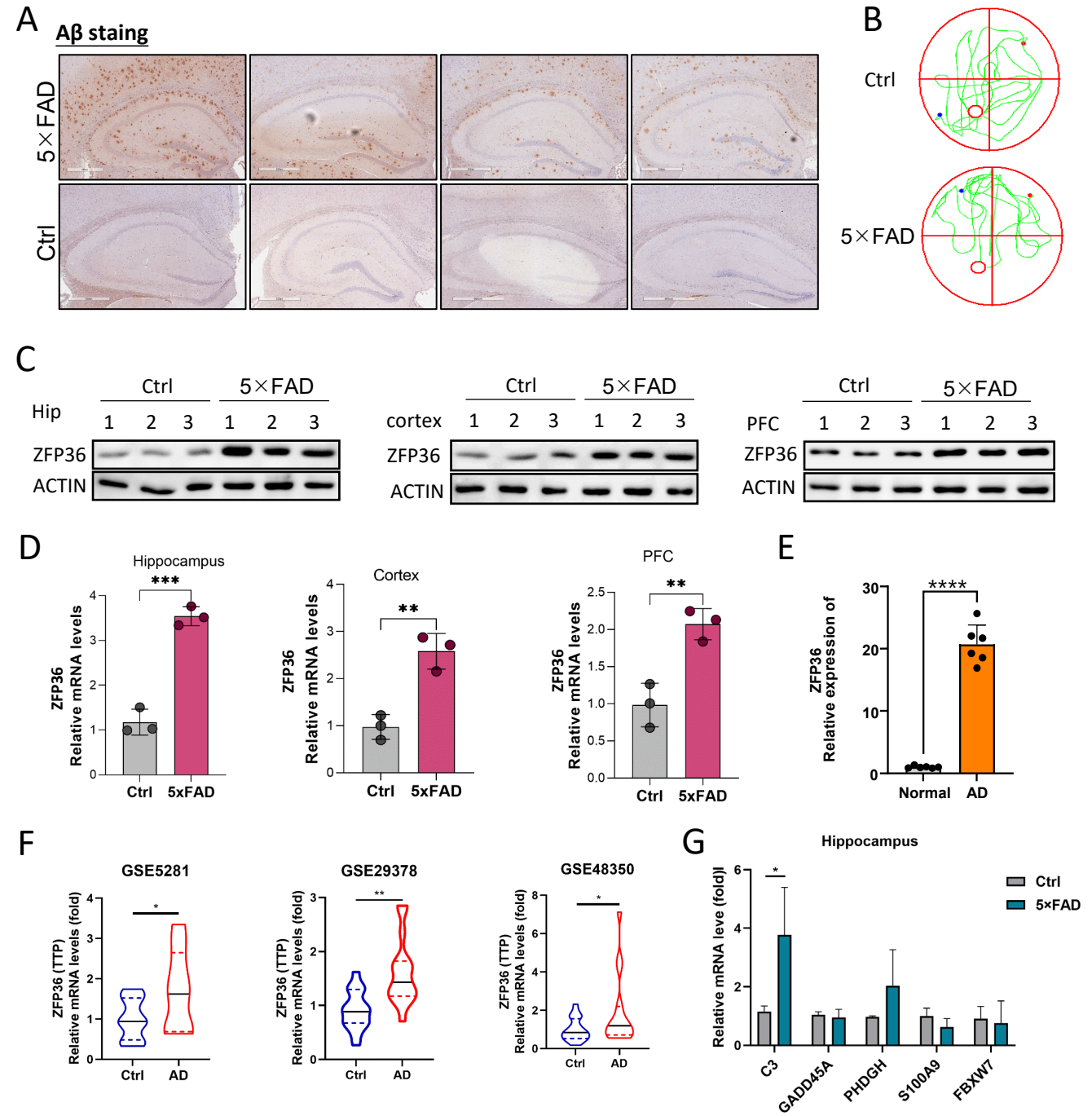


Figure 10.

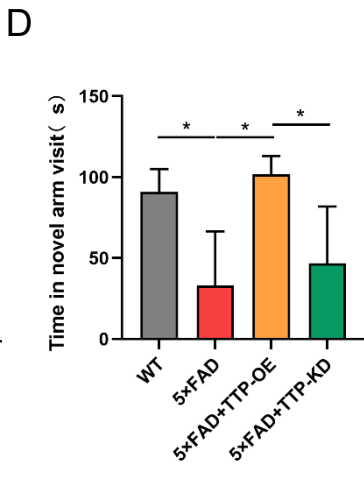
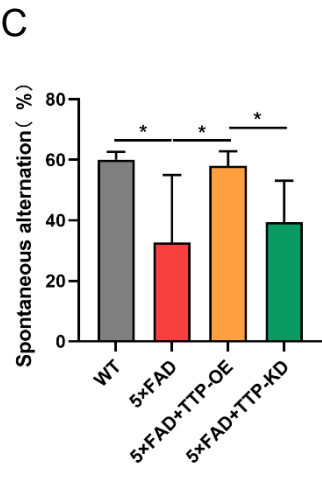
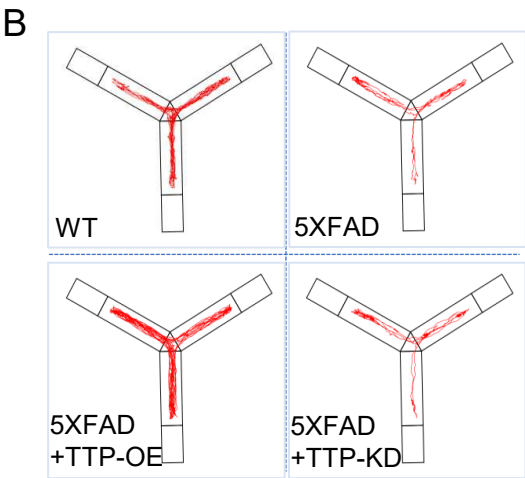
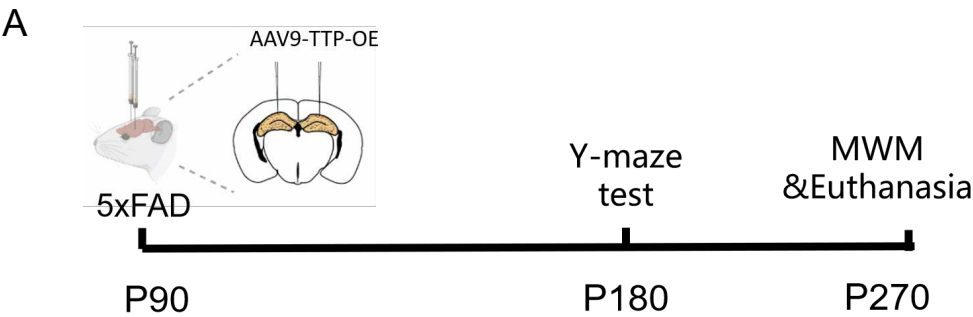
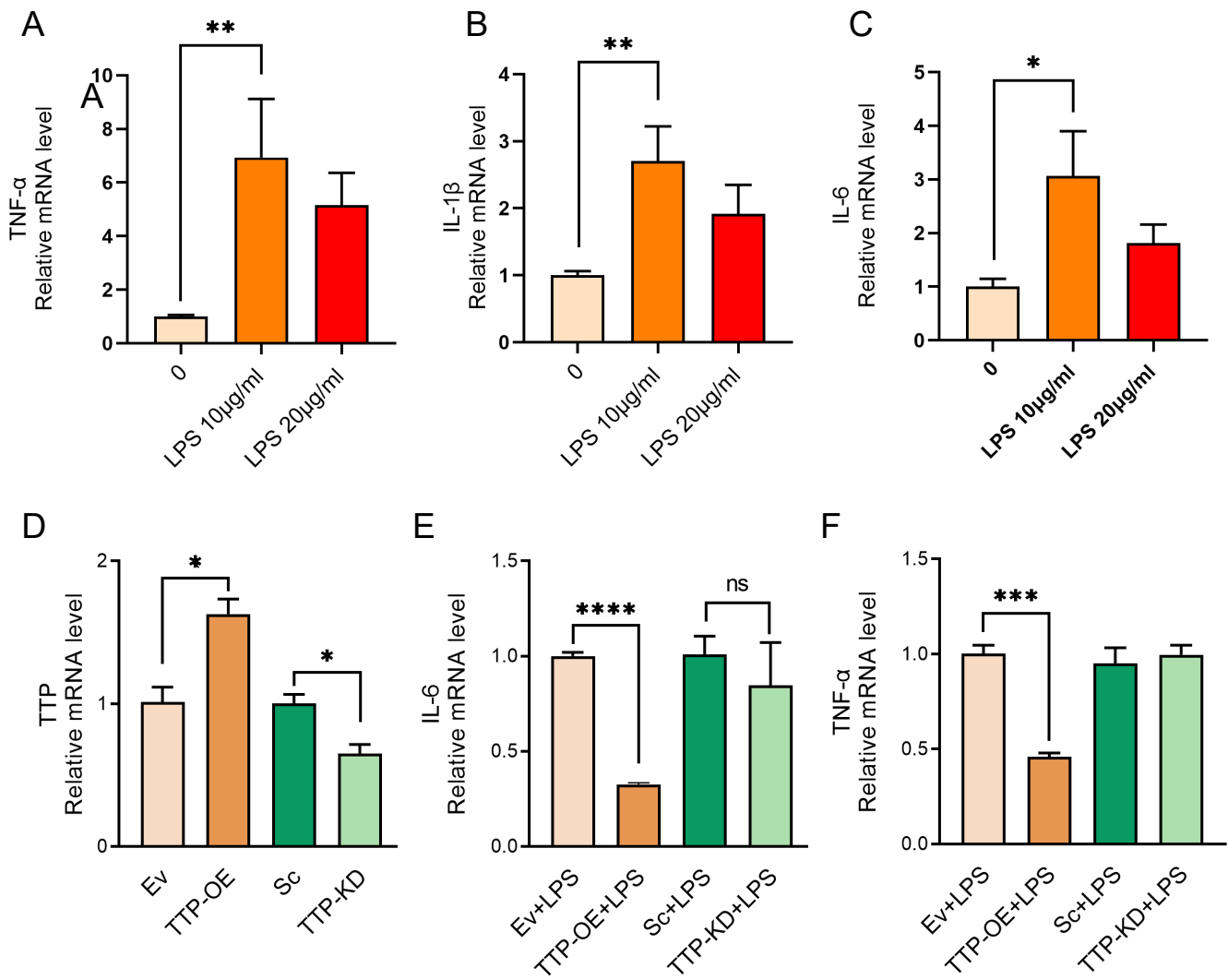


Figure 11.



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- [figureS.rar](#)