

The effects of Arf4 gene knockdown on Alzheimer's amyloid β deposition: Possible role in cognitive resilience

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Additional Declarations: Competing interest reported. Dr. Gandy is a co-founder of Recuerdo Pharmaceuticals. He has served as a consultant in the past for J&J, Diagenic, and Pfizer, and he currently consults for Cognito Therapeutics, GLG Group, SVB Securities, Guidepoint, Third Bridge, MEDACORP, Altpep, Vigil Neurosciences, Memory Garden, and Eisai. He has received research support in the past from Warner-Lambert, Pfizer, Baxter, and Avid. The authors declare no other competing interests.

Abstract

Background

Studying the factors involved in cellular and cognitive resilience is a crucial step toward designing preventive and targeted therapeutic approaches for Alzheimer's disease (AD).

Methods

Systematic analysis of the proteomic landscape in the advanced aging populations identified ADP ribosylation factor 4 (ARF4) as one of the key driver genes associated with resilience among the oldest-old (OO) non-demented individuals. To assess the effects of *Arf4* gene knockdown in AD animal models, we generated APP^{KM670/671NL} x PSEN1^{Δexon9} (PSAPP)/*Arf4*^{+/-} mice.

Results

Protein signatures and protein coexpression network modules of the oldest-old (OO) non-demented individuals were identified. Integrative protein network analysis further revealed ARF4 as a top protein driver of OO. Mouse experiments showed that *Arf4* knockdown was associated with increased amyloid plaque burden and enhanced microglial activity. The consequence of *Arf4* knockdown was an alteration in amyloid precursor protein (APP) localization, which led to variations in mature APP levels. *Arf4* knockdown further led to transcriptomic alterations in the mouse brain; most pronounced in pathways associated with immune regulation and microglial homeostasis.

Conclusion

Based on the exacerbated AD pathological features and transcriptomic alterations observed in *Arf4* knockdown animals, ARF4 appears to play a critical role in AD resilience and may represent a potential prognostic factor worthy of further investigation.

Background

Aging is recognized as one of the most significant factors associated with development and progression of dementia, especially Alzheimer's disease (AD) [1]. However, recent research suggests the concept that some of the "oldest old" (OO) individuals develop cellular resilience to AD pathology characterized by minimal to absent accumulation of amyloid β plaques and neurofibrillary tangles, and no synaptic loss [2]. Additionally, postmortem brain analyses have shown that some individuals have cognitive resilience to developing clinical symptoms despite having AD pathology [3]. Epidemiological studies investigating potential demographic factors associated with cognitive resilience have implicated sex, occupation, level of education, and mood [4, 5]. To date, there have been only limited investigations into molecular and

genetic factors that contribute to cellular resilience. Further research is required to identify prognostic factors and potential therapeutic targets.

The implicated molecules and pathways represent the same networks that are dysregulated in aging and AD, including peptides involved in synaptic plasticity (VGF, protachykinin-1) [6], brain metabolism [7], and bioenergetics (e.g., UCP4 [8] and ABCA7 [9]). However, previous studies did not account for different age groups in their omics research, particularly the non-demented OO who have lived beyond the age of greatest risk for AD and demonstrated both cellular and cognitive resilience [10].

In this study, we define cognitive resilience as OO without dementia. We first performed large-scale proteomic profiling of prefrontal cortex samples from 282 human postmortem brains from OO individuals from the Mount Sinai/James J Peters VA Medical Center Brain Bank (MSBB) and the Religious Orders Study/Rush Memory and Aging Project (ROSMAP). Integrative trend analysis and network construction revealed that the proteomic landscape was altered when samples from younger persons were compared to samples from the OO group. Our key driver analysis of the co-expression network showed potential regulators of cognitive resilience, with ADP-ribosylation factor 4 (ARF4) emerging as one of the notable candidates.

ARFs are a group of guanine nucleotide-binding proteins involved in important intracellular trafficking through the plasma membrane, endosome-lysosomal system, and Golgi complex [11]. Based on gene structure and protein size, ARFs are categorized into three groups as: class I (ARF1, -2, and -3), class II (ARF4 and -5), and class III (ARF6) [12]. The functions of different classes of ARFs have been reported to be similar in that all supplement the activation of phospholipase D (PLD). However, ARF4 has been reported to have unique intracellular interactions such as the EGFR-induced activation of PLD2 [11], Bcl-2-associated X protein (bax)-induced apoptosis [13], ciliary assembly through Notch signaling [14], and cerebral cortex development through N-cadherin trafficking [15]. The roles of ARF4 in disease have only rarely been investigated. For instance, ARF4 has been reported as an anti-apoptotic factor involved in glioblastoma progression [16, 17] and as an inducer of activator protein 1 (AP-1) in breast cancer cell migration [18]. There is limited information on the role of ARF4 in neurodegenerative disorders. To understand the effects of ARF4 in AD pathology, we developed *Arf4* knockdown mice to assess the effects of this protein on endogenous and transgenic APP at the molecular level.

Materials and methods

Proteomic profiling datasets: The MSBB and ROSMAP Cohort AD Resilience Study

The MSBB and ROSMAP cohort AD Resilience Study (Synapse ID: syn52676457 at <https://www.synapse.org/Synapse:syn52676457>) consists of human postmortem brain samples from two AD clinical cohorts: the Mount Sinai/James J Peters VA Medical Center Brain Bank (MSBB) and the Religious Orders Study/Rush Memory and Aging Project (ROSMAP), each containing 82 and 200

individuals, respectively. ROSMAP participants enrolled without known dementia [2]. All agree to annual clinical evaluation and brain donation at death. Both studies were approved by an Institutional Review Board of Rush University Medical Center. Each participant signed an informed consent, Anatomic Gift Act, and an RADC Repository consent allowing the data and biospecimens to be repurposed. Clinical diagnoses of Alzheimer's dementia, mild cognitive impairment (MCI), and no cognitive impairment (NCI) were made as described [19–21]. We conducted proteomic profiling on the proteomes of prefrontal cortex tissue derived from each of the 282 samples of persons without dementia [22]. A 10 mg portion of brain tissue from each brain was weighed, homogenized, and subjected to a protein quality examination by SDS gel electrophoresis [23]. The 282 samples were then randomized into 29 batches with respect to confounding factors (e.g., sex, age). For each batch, proteins were digested, labeled by tandem mass tag (TMT) reagents, and pooled for two-dimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS) analysis [24]. The first LC fractionation was performed to reduce sample complexity for improved protein identification, and the second LC was directly coupled with MS for peptide/protein identification and quantification. Further protein quantification, normalization, quality check (QC), batch adjustment, cross-data sample matching, and computation work are detailed [22].

After rigorous QC to remove samples with significant protein degradation, 265 samples were retained for further analysis (Supplementary table). Across the QC'd samples, we identified 11,803 unique protein isoforms (here is referred to as proteins) that span 9,462 genes, resulting in 1.25 protein isoforms per gene on average. The abundance of each protein was normalized, log2-transformed and further adjusted for the covariates including postmortem interval (PMI), race, gender, batch, and cohort using a mixed linear model[25] as $\text{lmer}(x \sim \text{PMI} + (1|\text{race}) + (1|\text{batch}) + (1|\text{cohort}) + (1|\text{sex}))$, where cohort is MSBB vs. ROSMAP. The residuals from the regression were used for downstream analysis.

Differential expression analysis

Differential expression analysis was performed by the moderated t-test implemented in the limma package [26]. The QC'd subjects were classified as oldest old (OO) with age at death (AOD) > 85 or else medium old (MO), and differentially expressed proteins (DEP) were defined as false discovery rate (FDR) < 0.05 in OO vs. MO. The DEPs were split into down-regulated (fold change < 0 in OO vs. OM) or otherwise up-regulated.

Correlation analysis

The Pearson's correlation between AOD and each protein across all the samples was calculated. Aging-correlated proteins were defined as the correlation with FDR < 0.05. The correlated proteins were split into negatively (correlation coefficient with AOD < 0) or otherwise positively correlated.

Multiscale embedded gene co-expression network analysis (MEGENA)

To reveal the complex interactions among proteins, we constructed the protein co-expression network using the profiled proteome via the MEGENA pipeline [27]. Briefly, Pearson correlation coefficients (PCCs) were computed for all protein pairs. Then, significant PCCs were filtered by an FDR cutoff of 0.05 with ten permutation analyses. The ranked significant PCCs were iteratively tested for planarity to grow a Planar Filtered Network (PFN) using the Planar Maximally Filtered Graph (PMFG) algorithm [27]. The resulted PFN was analyzed by Multiscale Clustering Analysis (MCA) to identify co-expression modules at different network scale topology.

We next ranked-order the modules using the ensemble approach as described in [28–30]. Briefly, we first split the DEP signature into down- and up-regulated DEPs in OO vs. OM. Similarly, aging-associated proteins were split into negatively and positively correlated proteins. We then calculated the enrichment p values (multiple-test adjusted) of each module for the DEP and aging-associated protein signatures via the hypergeometric test[31], which were subsequently aggregated into a single metric (p value) using the ACAT algorithm[30]. The modules were finally sorted over the aggregated p values, resulting in the rank-orders for each module with rank # 1 having the lowest p value.

We also conducted key driver analysis (KDA) to identify global hub proteins (GHP) by projecting the DEP signature over the MEGENA co-expression network using the method as described in [32, 33].

Construction of ARF4 subnetwork in the MEGENA co-expression protein network

The ARF4 subnetwork consists of the proteins in the ARF4's neighborhood (up to n hops) in the protein MEGENA co-expression network, where n is the hop (walk) away from ARF4 in the network[32]. Different numbers (n) of walks are often tested to achieve the ideal outcome. Duplicated proteins were made unique by keeping one of the isoforms where gene level analysis is applied.

Construction of ARF4-correlation centric protein network

Pearson's correlation between ARF4 and each of the other proteins in the human PFC proteome was calculated. The AFR4-correlation centric protein network was defined as the proteins with an absolute correlation coefficient > 0.2 and FDR < 0.05. Duplicated proteins were made unique by keeping one of the isoforms where gene level analysis is applied.

Analysis of Enrichr

The gene set libraries including GO_Molecular_Function_2023, GO_Cellular_Component_2023, GO_Biological_Process_2023 were utilized as databases, whereas the aging DEPs were used as input.

The enrichment analysis[34] was performed separately for down- and up-regulated aging DEPs, respectively. The enrichment of DEPs for gene sets was implemented using the function Enrichr in the R package Enrichr with default setting.

Animals

All procedures were conducted according to the National Institutes of Health (NIH) animal research guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at Icahn School of Medicine at Mount Sinai. APP^{KM670/671NL} x PSEN1^{Δexon9} (PSAPP), B6. Cg- *Arf4*^{tm1Gjp/J} (*Arf4*^{fl/fl}; stock no.: 030538), and B6.C-Tg (CMV-Cre) 1Cgn/J (germline CMV-Cre; stock no.: 006054) were purchased from The Jackson Laboratory. *Arf4*^{fl/fl} mice were crossed with PSAPP mice to obtain PSAPP; *Arf4*^{fl/fl} and their *Arf4*^{fl/fl} littermates. Constitutive *Arf4* knockdown mice were generated by crossing *Arf4*^{fl/fl} animals to CMV-Cre and then backcrossed to remove the CMV-Cre to obtain only *Arf4*^{+/-} (referred to as *Arf4*(KO) heterozygote) mice. All mice were on the C57BL/6J background.

Tissue collection and sample preparation. 9-month-old female mice were anesthetized in a CO2 chamber followed by 20 ml ice-cold phosphate buffered saline (PBS) perfusion. One hemisphere was immersed in paraformaldehyde (PFA) 4% for immunohistochemical studies and the other hemisphere was dissected to collect hippocampus and cortex which were snap frozen and stored at -80°C. Cortical samples were used for amyloid β and protein quantification, medial prefrontal cortex (mPFC) samples were used for quantitative PCR and hippocampi were stored for RNA sequencing.

Processing bulk RNA-seq for mouse hippocampus

Bulk RNA-sequencing was performed on hippocampus isolated from PSAPP;*Arf4*^{+/-} and PSAPP;*Arf4*^{+/+}, hereafter designated as PSAPP;*Arf4*(KO) heterozygote and PSAPP;*Arf4*(WT), respectively. Bulk RNA-seq was performed by Novogene (<https://en.novogene.com>) using Illumina Novaseq 6000 S4 flow cells. Read quality was evaluated via FastQC (v.0.11.5). The fastq reads were mapped back to the mouse reference genome (GRCm39) using the star package (v2.7.0e). Raw counts at the gene level over the annotation of Mus_musculus.GRCm39.104.gtf was acquired using the function featureCounts from the package subread (v1.5.2).

The detection of differentially expressed genes (DEG) was conducted using our in-house bulk RNA-seq processing pipeline[28] that is adapted from the limma-voom method[35–37] including gene filtering, normalization, voom transformation, and linear modeling. DEG analysis was only on the mouse genes that have a count per million (CPM) > 1 in more than 10% of the samples. The normalized count data was further adjusted for batch (sex) by random model, and then total reads per sample via linear model. Note here the mouse sex was used as batch since the first and second set of RNA-seq were carried out on female and male mice, respectively.

Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) was conducted using the R package clusterProfiler[38] with default setting. For Gene Ontology (GO) enrichment of differentially expressed genes (DEGs) from PSAPP;*Arf4*(KO) heterozygote samples, the gseGO function was employed. For mouse brain cell type[39] enrichment, as well as ARF4 correlation-centric and subnetwork enrichment analyses of PSAPP;*Arf4*(KO) DEGs, the GSEA function was applied.

For both GO and mouse brain cell type[39] enrichment analyses, the input gene list consisted of mouse DEGs filtered by a defined p-value threshold and ranked in descending order based on fold change (FC). For ARF4 protein subnetwork and correlation-centric network enrichment analyses, the input gene list included mouse DEGs filtered by a specified p-value threshold and $FC < -0.20$ and subsequently ranked in descending order according to $-\log_{10}(p\text{-value})$. Multiple cutoff thresholds (< 0.45) were tested, and the optimal threshold was selected based on enrichment performance.

Immunohistochemistry

Post-fixed hemispheres were cut into 30 μm coronal sections with a vibratome (Leica). After antigen retrieval with 70% formic acid, sections were washed with PBS and further incubated with 0.1% Triton in PBS/5% goat serum for one hour to eliminate non-specific binding. Then, the sections were incubated with rabbit anti-Iba1 (1:500, cat#019-19741, Wako) and mouse 6E10 anti-A β (1:1000, cat#9320 – 500, Covance) overnight. Probed sections were incubated with anti-rabbit Alexa 488 (1:400, cat#A-11008, ThermoFisher Scientific) and anti-mouse Alex 594 (1:400, cat#A-11005, ThermoFisher Scientific). For amyloid β and TGN38 co-immunostaining, the same protocol was followed using rabbit H31L21 anti-A β (cat#700254, ThermoFisher Scientific) and TGN38 mouse antibody (1:500, cat#NB300, Novus Biological). Olympus BX61 microscope was used to obtain images. All the image analyses were performed using Fiji software (ImageJ).

A β ELISA

Cortical samples were homogenized in TBS/EDTA homogenizing buffer containing protease/phosphatase inhibitor cocktail (cat#78443, Pierce). A 250 μl aliquot of the homogenates were used for serial detergent fractionation with ultracentrifugation, and the rest of the homogenates were stored at -80°C and later mixed with RIPA buffer for western blotting. After the first round of ultracentrifugation at 100,000 $\times g$ for 1 hour at 4°C , the TBS soluble supernatant was collected for quantification of soluble amyloid β (A β) peptide in PSAPP samples, and the pellet was subsequently homogenized in Triton-X solution (TBS with 1% Triton-X-100) with a protease and phosphatase inhibitor cocktail and similarly ultra-centrifuged. Triton-X soluble supernatant was collected, and the pellet was homogenized in 70% formic acid for the final round of ultracentrifugation. The formic acid (FA) fraction was further neutralized with 1 M Tris buffer, PH 11. This fraction was used for quantification of insoluble A β in transgenic mice. Human/rat A β_{1-40} and A β_{1-42} ELISA kits (Wako, cat#294-64701 and \$#290-62601) were used for A β level quantification according to the manufacturer's instruction. Final quantifications of A β were normalized to initial tissue weight.

Western blots

Cortical homogenates were re-homogenized in RIPA buffer (ThermoFisher Scientific) with phosphatase/protease inhibitor cocktail (cat#78443, Pierce) and subsequently centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatant was collected and 20µg aliquots of total protein were separated by electrophoresis in precast 4% to 12% Bis-Tris Gels (Bio-Rad) and transferred to PVDF membranes. After blocking the membranes with 5% milk, The membranes were probed with the following primary antibodies: rabbit anti-PSD95 (1:1000, cat#2507, Cell Signaling), rabbit anti-synaptophysin (1:1000, cat#4329, Cell Signaling), mouse 6E10 anti-Aβ (1:1000, cat#9320 – 500, Covance), rabbit 369 anti-APP C-terminus polyclonal antibody (1:1000, generated in our laboratory), mouse anti-GAPDH (1:1000,). For investigation of variations in posttranslational modifications (PTM) of APP, each solution of 20µg total protein extracts was incubated with 2µl N-glycosidase F (cat# 11365169001, Sigma-Aldrich) overnight at 37°C to de-glycosylate proteins. The reaction was terminated by adding Laemmli sample buffer (Bio-Rad) and incubating at 95°C for 5 minutes. Following separation by electrophoresis with the same Bis-Tris gel system and transfer to nitrocellulose, the membranes were blocked with 5% milk for one hour followed by probing with rabbit 369 anti-APP, mouse 6E10 anti-Aβ and mouse anti-GAPDH (all 1:1000). The secondary antibodies were as follows: peroxidase-labeled anti-mouse IgG (1:2000, Vector Laboratories), peroxidase-labeled anti-rabbit IgG (1:2000, Vector Laboratories). Enhanced chemiluminescence (ECL) (#32106, Pierce) was used to reveal immunoreactive proteins, and images were obtained using a BioRad ChemiDoc imaging system. Membranes were stripped with stripping buffer (cat#46430, Thermo Scientific) for additional antibody probing when needed. Optical densities of protein bands were quantified using Fiji (ImageJ). The protein levels of samples were normalized to their corresponding GAPDH bands.

RNA extraction and qPCR analysis

RNA was isolated from mouse mPFC using the QIAzol® Lysis Reagent (Qiagen) and the miRNeasy® Micro Kit (Qiagen). 250 ng of total RNA was reverse transcribed using the High-Capacity RNA-to-cDNA Kit (ThermoFisher, #4387406). cDNAs were subjected to real-time qPCR in a StepOne Plus system (Applied Biosystem) using The All-in-One qPCR Mix (GeneCopoeia, #QP001-01). Sequences of oligonucleotides used were: Mm99999915_g1_Gapdh (4331182, ThermoFisher) and Mm00431854 m1 ARF4 4331182, ThermoFisher). The individual value for each test transcript, performed in triplicate, was normalized to the abundance of GAPDH. Relative quantification was performed using the $\Delta\Delta CT$ method and was expressed as fold-change relative to control by calculating $2^{-\Delta\Delta CT}$.

Statistical analysis.

Molecular data for mice were analyzed with GraphPad Prism 9. One way ANOVA and student's t-test were used for parametric tests. Non-parametric Mann-Whitney tests were used when normal distribution of the samples was not determined.

Results

Study design and data preprocessing

The present study aims to define the molecular architecture of healthy brain aging and identify proteomic signatures associated with resilience to AD using TMT-based quantitative proteomics of postmortem prefrontal cortex tissue from cognitively healthy human cohorts. Following stringent QC, covariate normalization, differential expression, and MEGENA-based co-expression and key driver analyses, we identified ARF4 as a candidate hub protein associated with healthy aging and further investigated its neuroprotective potential in the PSAPP transgenic mouse model of AD (Fig. 1).

Identification and characterization of aging-associated proteins.

We conducted differential expression analysis to identify proteins associated with aging. Participants were stratified into two age-defined groups: individuals aged > 85 years were classified as the oldest-old (OO) group, whereas the remaining participants were designated as the moderately old (MO) group (see the **Methods and Materials**). In total, 671 differentially expressed proteins (DEPs) were identified in OO vs. MO with an adjusted p-value < 0.05. Among these, 560 proteins were down-regulated and 111 were up-regulated in OO relative to MO (Fig. 2A). The markedly downregulated DEPs include ARF4, GNAI1, GNAI3, and DYNLRB1, whereas NSUN5, IFT140, CYP4F2, REM2, and KIF9 were among the prominently upregulated proteins (Fig. 2A; Supplementary table 1). We also performed correlation analysis between each protein and age and intersected age-correlated proteins (FDR < 0.05) with DEPs, which showed significant overlaps in the same directionality. Thus, we identified a substantial number of proteins that are relevant in aging.

We next explored the DEPs for biological significance by querying their enrichment for the gene ontology (GO) terms including biological processes (BP), cellular component (CC), and molecular functions (MF) (see the **Methods and Materials**). We detected 230 significant GO terms, among which 218 and 12 are for the down- and up-regulated DEPs, respectively (Supplement table). Figure 2B highlighted top ranked GO terms such as protein localization, vesicle-mediated transport, axon guidance, protein modification process, and neuron projection guidance for down-regulated DEPs, and nuclear lumen, nucleolus, and MHC protein complex for up-regulated DEPs, respectively (Fig. 2B). Since many of the GO terms for down-regulated DEPs are related to neuronal functions (Fig. 2B, and Supplement table), we further examined the down-regulated DEPs in the context of synaptic location by querying the SynGO[40] database. The down-regulated DEPs are most enriched for synaptic and presynaptic terms (Fig. 2C).

We further inspected the concordance between aging-associated proteins and genes. We acquired the aging-associated gene signatures for human brain cortex and hippocampus[41], and then tested the significance of their overlapping with our DEP signatures. The down-regulated DEPs are significantly overlapped with the down-regulated DEGs in aging for both human cortex (fold enrichment (FE) = 2.8, FDR = 2.4e-12) and hippocampus (FE = 2.1, FDR = 0.001) (Fig. 2D). Similarly, the up-regulated DEPs are significantly overlapped with the up-regulated DEGs in aging for human hippocampus (FE = 2.7, FDR = 0.02) (Fig. 2D). Thus, these results validated the relevance of our DEPs in aging.

Multiscale embedded gene co-expression network analysis (MEGENA).

To reveal the structures of complex interactions among the proteins in healthy brain prefrontal cortex, we constructed a protein co-expression network through the MGENEA[27] algorithm over the entire profiled proteome (Fig. 3A). We detected 474 co-expressed network modules with an average of 88 in module membership size, ranging from 10 to 4084 (Fig. 3B, and Supplementary table). GO analysis revealed that the co-expression modules were enriched for diverse biological processes, for example, immune response, synaptic signaling, mitochondrial translation, protein folding, cellular metabolic process, and microtubule-based process (Figure S2, and Supplementary tables 2–4). Furthermore, the network modules are enriched for different human brain cell types[39]. For instance, modules M3, M7, M8, M57, and M314 are enriched for marker genes of oligodendrocyte, microglia, neuron, endothelial, and astrocytes (Fig. 3B).

To identify co-expression modules that are associated with aging, we assessed the significance of the intersections between each MGENEA module and our DEPs and age-correlated protein signatures. Then, we aggregated the enrichment p values into an overall enrichment score[28, 42] to quantify the association strength of each module with aging, thus enabling rank-ordering of the modules in relevance to aging. Figure 3C summarizes the enrichment for each aging-associated protein signature, and overall module ranking scores of the top 30 modules. M4 is most associated with aging (Fig. 3C), followed by M38, M418, and M147, all of which are daughter modules to M4 (Supplementary table 5). Overall, M4 is down-regulated in aging as it contains many down-regulated DEP nodes (Fig. 3D). Among the biological processes, it is most enriched for protein folding and microtubule-based processes.

Next, we sought to identify and prioritize global hub proteins (GHP)[33] in the co-expression protein network using the key driver analysis (KDA)[28, 32, 42]. We projected our aging related DEP signature onto the protein co-expression network to identify GHPs (see the details in the **Methods and Materials**). We identified 129 GHPs over the entire network (**Supplementary Table 6**), out of which 111 are within the M4 module (Fig. 3D). A vast majority (72%) of the GHPs in M4 are also downregulated during aging (Fig. 3C). Notably, ARF4 is one of the top five GHPs in M4 that are also associated with ageing (Fig. 3C).

Prioritization of key regulatory proteins, network validation and integration

To identify key regulatory proteins for experimental validation, we prioritized candidates using three stringent criteria: (1) designation as a top-ranked differentially expressed protein (DEP) during aging which are the 20 DEPs with the lowest FDR; (2) classification as a primary global hub protein (GHP) within the top-ranked aging module M4; and (3) availability of established mouse models for genetic perturbation.

ARF4 met all selection criteria: it is a top-ranked DEP that is downregulated during aging (Fig. 2A), a GHP within module M4 (Fig. 3D). Therefore, we chose ARF4 for further experimental validation to directly investigate its functional role in aging-related processes.

We began by examining transcriptome-wide changes following *Arf4* perturbation in mouse hippocampus. We identified 393 differentially expressed genes (DEGs) at a significance cutoff of $p < 0.05$ in PSAPP;*Arf4*^{+/-} relative to PSAPP;*Arf4*(WT) mice (Fig. 4C). Notably, *Arf4* exhibited the most pronounced reduction in expression in PSAPP;*Arf4*^{+/-} mice, with an approximate 30% decrease ($p = 3.2e-7$) (Fig. 4B, left panel Supplementary Table 7). Furthermore, a substantial proportion of microglia-specific genes [40] were downregulated following *Arf4* knockdown in the PSAPP background (Supplementary Table 7). For instance, expression of *P2ry12* was significantly reduced in PSAPP;*Arf4*^{+/-} compared to PSAPP;*Arf4*(WT) mice ($p = 0.003$) (Fig. 4B, right panel). Consistent with this, GO biological processes enriched for the differentially expressed genes (DEGs) largely involve immune functions that are suppressed following *Arf4* knockdown (Fig. 4C). The DEGs also exhibited negative enrichment for mouse microglial marker genes (Fig. 4D), suggesting that microglia-associated transcriptional programs might be suppressed following *Arf4* knockdown.

Next, we projected the mouse PSAPP;*Arf4*(KO) DEG signature onto the ARF4-protein subnetwork (see **Methods and Materials**) for GSEA analysis. We found a significant enrichment of the mouse DEG in the ARF4-protein subnetwork neighborhood (normalized enrichment score (NES) = 1.74, $p_{\text{adj}} = 0.0097$, Fig. 4E). Similarly, the mouse PSAPP;*Arf4*(KO) DEG signature was also enriched in the ARF4-correlation centric protein network (NES = 1.84, $p_{\text{adj}} = 0.0019$, Fig. 4F). These results converged to strongly support a key role of ARF4 on its co-expression neighborhood and thus validate the co-expression protein network structure.

Arf4 knockdown in PSAPP mice is associated with increased amyloid plaque formation and microglial recruitment.

Effects of ARF4 gene knockdown on A β deposition were assessed by measuring plaque density in hippocampus of female PSAPP mice. The percentage of 6E10 + area PSAPP;*Arf4*(KO) samples was twice that observed PSAPP;*Arf4*(WT) brains ($p = 0.0087$). The total number of Iba1 + microglia, as well as plaque-associated microglia, was also increased in the hippocampus of PSAPP;*Arf4*^{+/-} samples ($p < 0.0001$) (Fig. 5A). Plaque-associated Iba1 + microglia were also significantly increased in PSAPP;*Arf4*^{+/-} animals indicating greater recruitment of microglia to amyloid deposits ($p = 0.0192$) (Fig. 5B). These results indicate increased amyloidosis and inflammation in PSAPP;*Arf4*^{+/-} mice.

Arf4 knockdown alters subcellular localization of APP.

To investigate the possible mechanisms of *Arf4* gene knockdown in increased plaque deposition, we used high power microscopy of 6E10 stained slides to assess the subcellular localization of APP. Considering previous studies as a reference for possible localization of APP, PSAPP;*Arf4*(WT) samples show a regular distribution as mostly compatible with Golgi complex whereas PSAPP;*Arf4*^{+/-} samples showed predominantly lysosomal distribution which has been reported as a main proteolytic mechanism for APP catabolism (Fig. 6A). In PSAPP;*Arf4*(WT) animals, H31L21-positive areas were mostly co-localized with TGN38-positive areas which is in line with previous studies on subcellular APP localization

[43]. However, there was a discrepancy between TGN38 and H31L21 positive areas in PSAPP;*Arf4*^{+/-} samples suggesting an alteration of APP sorting from its typical Golgi complex/lysosomal distribution. These findings suggest that ARF4 may alter lysosomal function and lead to increased APP accumulation (Fig. 6B). Lysosomal disruption has been reported to increase plaque burden [43]. To validate the reduction of ARF4 expression, we performed qPCR on mPFCs of the same animals which showed a significant decrease (about 50%) in relative expression (Fig. 6C).

Alterations in synaptic protein levels in *Arf4* knockdown wild type mice resemble those observed in PSAPP transgenic mice.

To assess the possible alterations in APP level, protein levels of female cortical samples were studied by western blotting using 6E10, specific to human, and 369, recognizing both human and mouse, antibodies. There was an alteration of mature APP protein level in PSAPP;*Arf4*(KO) heterozygote samples indicating that ARF4 deficiency altered the post-translational modification of endogenous APP (Figs. 7A, 7G).

To investigate possible roles of ARF4 gene in synaptic dysregulation, we studied levels of synaptophysin and PSD95 protein by western blotting. Synaptophysin levels were similar between PSAPP transgenic samples and *Arf4*(KO) heterozygote animals with only endogenous presenilin and APP, and significantly different from wild type animals. There was a significant increase in PSD95 levels in *Arf4*(KO) heterozygote samples versus PSAPP;*Arf4*(WT) samples. Altogether, our results show that *Arf4* gene protein product may have substantial roles in regulating endogenous APP metabolism and synaptic function (Figs. 7A, 7C-H). Notably, *Arf4*^{+/-} mice exhibit an abnormal phenotype even in the absence of PSAPP, indicating that heterozygous loss of *Arf4* results in haploinsufficiency.

***Arf4* knockdown decreases the level of mature APP, revealing a possible role for altered glycosylation**

Since APP is reported to undergo posttranslational modification by glycosylation, we next assessed glycosidase-treated samples to look for potential mechanisms underlying the altered protein banding pattern we observed when antibody 369 was used to detect APP-like immunoreactive protein bands from the brains of *Arf4*(KO) haploinsufficient mice. Western blotting showed that *Arf4*(KO) haploinsufficient samples showed a similar protein banding pattern to PSAPP transgenic mice whereas wild type animals showed cleaved APP bands. (Fig. 7B).

***Arf4* knockdown increases the amount of soluble amyloid β in wild type animals.**

A β levels in the FA fraction of PSAPP brain tissue (representing aggregated A β) did not show a significant difference between PSAPP;*Arf4*(KO) heterozygote and PSAPP;*Arf4*(WT) samples. However, soluble A β was significantly higher in *Arf4*(KO) haploinsufficient mice in comparison to wild type. This result is compatible with ARF4 modulation of endogenous amyloid metabolism as observed in immunoblotting. (Fig. 7I)

Discussion

Since OO population undergoes a normal aging process, the mechanisms underlying how they maintain cognitive health are a paramount question. By comparing the age-related proteomic changes with the AD-related proteomic changes, we defined the signatures for AD pathology and resilience in OO. Key driver analysis of the whole co-expression network identified potential regulators of cognition resilience. ARF4 was one of the key driver proteins that had not been evaluated in previous functional studies or confirmed by cognitive trajectory analysis and requires further experimental validations.

RNA sequencing identified *P2ry12* as one of the most significantly downregulated genes in PSAPP;*Arf4*(KO) mouse brains. P2Y12R is a purinergic receptor canonically expressed in surveillant microglia, where it mediates process extension toward sites of injury as part of the homeostatic damage-sensing response. In AD, P2Y12R-positive microglia associate with immature amyloid deposits characteristic of early disease, whereas mature compact plaques are surrounded by P2Y12R-negative microglia, reflecting progressive loss of microglial homeostasis as pathology advances [44, 45]. Interestingly, resilient brains show a higher number of microglia surrounding immature plaques compared to AD brains, suggesting that early and efficient microglial engagement at nascent deposits may serve as an effective defense mechanism against disease progression [46]. Therefore, the downregulation of *P2ry12* in PSAPP;*Arf4*(KO) heterozygote mice suggests that ARF4 may play a role in maintaining microglial homeostasis, and further studies are required to elaborate the mechanism underlying *Arf4* and P2Y12R association in transitioning the homeostatic microglia to disease-associated microglia.

We report that loss of ARF4 in PSAPP mice leads to an increase in plaque burden and enhances the recruitment of microglia to the plaques. Our findings align with previous reports on the role of ARF4 in protecting against dendritic spine loss induced by apoE4, a major genetic risk factor for AD [47]. As observed in our *study*, *Arf4* knockdown is linked to a reduction in synaptophysin levels, further supporting the potential involvement of ARF4 downregulation in significant synaptic loss, such as that seen in AD.

Given that the glycosylation pattern did not change in N-glycosidase-treated *Arf4*(KO) haploinsufficient samples compared to wild type, we hypothesize that ARF4 plays a role in reducing the extent of APP glycosylation. This has been previously linked to higher intracellular APP levels versus plasma membrane levels, as well as increased secreted A β , which correlates with exacerbated AD pathology, all of which are features of the *Arf4*(KO) haploinsufficient brains [48]. Additionally, reduced trafficking to the plasma membrane has been associated with increased endocytosis from the plasma membrane. Aging and genetic factors related to AD have been reported to contribute to enhanced endocytosis of APP [49]. This increase in APP processing can lead to the overproduction of A β_{42} [50]. This shift may compromise microglial phagocytic capacity and further exacerbate amyloid burden, consistent with our observation of significant enrichment of microtubule formation-related GO terms in PSAPP;*Arf4*(KO) heterozygote mouse brains. These data suggest a broader disruption of cytoskeletal dynamics that may underlie impaired microglial motility and plaque engagement.

Considering our results on the lysosomal distribution of APP in PSAPP;*Arf4*(KO) heterozygote brains and the role of glycosylation in APP trafficking, the underlying effects of ARF4 on APP glycosylation are highlighted. In addition, ARF4 has been shown to be involved in retrograde transport from endosomes to the TGN, which could explain the delocalization of APP from the TGN to lysosomes in PSAPP;*Arf4*(KO) heterozygote samples [51, 52]. The lack of a significant difference in APP levels between PSAPP;*Arf4*(KO) and PSAPP;*Arf4*(WT) animals may be attributed to the already substantial amyloid burden in these mice. Further investigation into the effects of ARF4 on amyloid plaque morphology using transmission electron microscopy (TEM) could provide insight into the aggregate ultrastructure and reveal potential mechanisms underlying the exacerbated AD pathology observed in PSAPP;*Arf4*(KO) heterozygote brains. It is interesting to note that patterns of synaptic disruption observed by western blotting and the quantity of amyloid were altered by ARF4 only in *Arf4*(KO) haploinsufficient and wild type animals rather than PSAPP transgenic mice. This highlights the role of ARF4 in resilience to AD rather than its pathogenesis.

Conclusion

Altogether, these findings suggest ARF4 as a novel factor associated with AD resilience, as its knockdown appears to disrupt endocytic trafficking of APP and compromise homeostatic microglial function, ultimately lowering the threshold for disease vulnerability and accelerating AD pathological progression.

Declarations

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Acknowledgments

Not applicable.

Authors' contributions

ME and BZ conceived the concept and designed the study. BX and JP performed the proteomics analysis. EW performed bioinformatic analyses. SH conducted mouse experiments. SG co-supervised animal studies. SH and EW wrote the initial draft. All the authors reviewed and revised the manuscript.

Conflict of interest

Dr. Gandy is a co-founder of Recuerdo Pharmaceuticals. He has served as a consultant in the past for J&J, Diagenic, and Pfizer, and he currently consults for Cognito Therapeutics, GLG Group, SVB Securities, Guidepoint, Third Bridge, MEDACORP, Altpep, Vigil Neurosciences, Memory Garden, and Eisai. He has

received research support in the past from Warner-Lambert, Pfizer, Baxter, and Avid. The authors declare no other competing interests.

Data and code availability

The proteomics data presented herein have been deposited onto the Accelerating Medicines Partnership- Alzheimer's Disease (AMP-AD) Knowledge portal with a synapse id of syn52676457. The AD Knowledge portal is a platform for accessing data, analyses, and tools generated by AMP-AD Target Discovery Program and other NIA-supported programs to enable open-science practices and accelerate translational learning. The data, analyses, and tools are shared early in the research cycle without a publication embargo on secondary use. Data are available for general research use according to the following requirements for data access and data attribution (<https://adknowledgeportal.synapse.org/DataAccess/Instructions>). The bulk RNA-seq dataset for the mouse brain hippocampus has been deposited in Synapse (syn77038910). The codes used in this study will be available via the repository of https://github.com/wange230/Resilience_proteomics.git at publication.

References

1. Yap LE, Hunt JE, Turner RS. Aging as a target for the prevention and treatment of Alzheimer's disease. *Front Neurol.* 2024;15:1376104.
2. Bennett DA, et al. Religious Orders Study and Rush Memory and Aging Project. *J Alzheimers Dis.* 2018;64(s1):S161–89.
3. Arenaza-Urquijo EM, et al. Sex and gender differences in cognitive resilience to aging and Alzheimer's disease. *Alzheimers Dement;* 2024.
4. Arenaza-Urquijo EM, et al. Sex and gender differences in cognitive resilience to aging and Alzheimer's disease. *Alzheimers Dement;* 2024.
5. Weisenbach SL, et al. Linking late life depression and Alzheimer's disease: mechanisms and resilience. *Curr Behav Neurosci Rep.* 2019;6(3):103–12.
6. Morgan GR, Carlyle BC. Interrogation of the human cortical peptidome uncovers cell-type specific signatures of cognitive resilience against Alzheimer's disease. *Sci Rep.* 2024;14(1):7161.
7. Arenaza-Urquijo EM, et al. The metabolic brain signature of cognitive resilience in the 80+: beyond Alzheimer pathologies. *Brain.* 2019;142(4):1134–47.
8. Montesanto A, et al. The Genetic Variability of UCP4 Affects the Individual Susceptibility to Late-Onset Alzheimer's Disease and Modifies the Disease's Risk in APOE- ϵ 4 Carriers. *J Alzheimers Dis.* 2016;51(4):1265–74.
9. Sassi C, et al. ABCA7 p.G215S as potential protective factor for Alzheimer's disease. *Neurobiol Aging.* 2016;46:e2351–9.

10. Haroutunian V, et al. Role of the neuropathology of Alzheimer disease in dementia in the oldest-old. *Arch Neurol*. 2008;65(9):1211–7.
11. Kim S-W, et al. ADP-ribosylation Factor 4 Small GTPase Mediates Epidermal Growth Factor Receptor-dependent Phospholipase D2 Activation*. *J Biol Chem*. 2003;278(4):2661–8.
12. Moss J, Vaughan M. Structure and Function of ARF Proteins: Activators of Cholera Toxin and Critical Components of Intracellular Vesicular Transport Processes (*). *J Biol Chem*. 1995;270(21):12327–30.
13. Woo IS, et al. Identification of ADP-ribosylation factor 4 as a suppressor of N-(4-hydroxyphenyl)retinamide-induced cell death. *Cancer Lett*. 2009;276(1):53–60.
14. Ezratty EJ, Pasolli HA, Fuchs E. A Presenilin-2-ARF4 trafficking axis modulates Notch signaling during epidermal differentiation. *J Cell Biol*. 2016;214(1):89–101.
15. Hara Y et al. ADP Ribosylation Factor 4 (Arf4) Regulates Radial Migration through N-Cadherin Trafficking during Cerebral Cortical Development. *eNeuro*, 2023. 10(11).
16. Budhiraja S, et al. ARF4-mediated retrograde trafficking as a driver of chemoresistance in glioblastoma. *Neuro Oncol*. 2024;26(8):1421–37.
17. Deretic D, Lorentzen E, Fresquez T. The ins and outs of the Arf4-based ciliary membrane-targeting complex. *Small GTPases*. 2021;12(1):1–12.
18. Jang SY, Jang S-W, Ko J. Regulation of ADP-ribosylation factor 4 expression by small leucine zipper protein and involvement in breast cancer cell migration. *Cancer Lett*. 2012;314(2):185–97.
19. Bennett DA, et al. Neuropathology of older persons without cognitive impairment from two community-based studies. *Neurology*. 2006;66(12):1837–44.
20. Bennett DA, et al. Natural history of mild cognitive impairment in older persons. *Neurology*. 2002;59(2):198–205.
21. Bennett DA, et al. Decision rules guiding the clinical diagnosis of Alzheimer's disease in two community-based cohort studies compared to standard practice in a clinic-based cohort study. *Neuroepidemiology*. 2006;27(3):169–76.
22. Wang E, et al. A Multi-Omics Study of Cognitive Resilience to Alzheimer's Disease. *bioRxiv*. 2026. 10.64898/2026.09.02.746746v1. <https://www.biorxiv.org/content/>.
23. Bai B, et al. U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2013;110(41):16562–7.
24. Niu M, et al. Extensive Peptide Fractionation and y(1) Ion-Based Interference Detection Method for Enabling Accurate Quantification by Isobaric Labeling and Mass Spectrometry. *Anal Chem*. 2017;89(5):2956–63.
25. Bates D, Bolker MM, Walker B. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw*. 2015;67(1):48.
26. Okonkwo OC, Vemuri P. Stemming the Alzheimer tsunami: introduction to the special issue on reserve and resilience in Alzheimer's disease. *Brain Imaging Behav*. 2017;11(2):301–3.

27. Song WM, Zhang B. Multiscale Embedded Gene Co-expression Network Analysis. *PLoS Comput Biol.* 2015;11(11):e1004574.
28. Wang M, et al. Transformative Network Modeling of Multi-omics Data Reveals Detailed Circuits, Key Regulators, and Potential Therapeutics for Alzheimer's Disease. *Neuron.* 2021;109(2):257–e27214.
29. Wang E, et al. Genome-wide methylomic regulation of multiscale gene networks in Alzheimer's disease. *Alzheimers Dement*; 2023.
30. Liu Y, et al. ACAT: A Fast and Powerful p Value Combination Method for Rare-Variant Analysis in Sequencing Studies. *Am J Hum Genet.* 2019;104(3):410–21.
31. Rivals I, et al. Enrichment or depletion of a GO category within a class of genes: which test? *Bioinformatics.* 2007;23(4):401–7.
32. Zhang B. J.Z., *Identification of Key Causal Regulators in Gene Networks*. Proceedings of the World Congress on Engineering, 2013. II: p. 4.
33. Shrestha HK et al. Pan-neurodegeneration proteomics reveals disease subtypes and molecular signatures. *Cell*, 2026.
34. Chen EY, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics.* 2013;14:128.
35. Law CW et al. RNA-seq analysis is easy as 1-2-3 with limma, Glime and edgeR. *F1000Res*, 2016. 5.
36. Ritchie ME, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43(7):e47.
37. Law CW, et al. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.* 2014;15(2):R29.
38. Yu G, et al. clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics.* 2012;16(5):284–7.
39. McKenzie AT, et al. Brain Cell Type Specific Gene Expression and Co-expression Network Architectures. *Sci Rep.* 2018;8(1):8868.
40. Koopmans F, et al. SynGO: An Evidence-Based, Expert-Curated Knowledge Base for the Synapse. *Neuron.* 2019;103(2):217–e2344.
41. Gonzalez-Velasco O, et al. Transcriptomic landscape, gene signatures and regulatory profile of aging in the human brain. *Biochim Biophys Acta Gene Regul Mech.* 2020;1863(6):194491.
42. Wang E, et al. Multiscale proteomic modeling reveals protein networks driving Alzheimer's disease pathogenesis. *Cell.* 2025;188(22):6186–e620413.
43. Caporaso GL, et al. Morphologic and biochemical analysis of the intracellular trafficking of the Alzheimer beta/A4 amyloid precursor protein. *J Neurosci.* 1994;14(5 Pt 2):3122–38.
44. Maeda J, et al. Distinct microglial response against Alzheimer's amyloid and tau pathologies characterized by P2Y12 receptor. *Brain Commun.* 2021;3(1):fcab011.
45. Smith JA, et al. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Res Bull.* 2012;87(1):10–20.

46. Jury-Garfe N, et al. Enhanced microglial dynamics and a paucity of tau seeding in the amyloid plaque microenvironment contribute to cognitive resilience in Alzheimer's disease. *Acta Neuropathol.* 2024;148(1):15.
47. Jain S, et al. Arf4 determines dentate gyrus-mediated pattern separation by regulating dendritic spine development. *PLoS ONE.* 2012;7(9):e46340.
48. Lin T, et al. Lack of N-glycosylation increases amyloidogenic processing of the amyloid precursor protein. *Glycobiology.* 2022;32(6):506–17.
49. Zadka Ł, et al. Endocytosis and Alzheimer's disease. *Geroscience.* 2024;46(1):71–85.
50. Alsaqati M, Thomas RS, Kidd EJ. Upregulation of endocytic protein expression in the Alzheimer's disease male human brain. *Aging Brain.* 2023;4:100084.
51. Nakai W, et al. ARF1 and ARF4 regulate recycling endosomal morphology and retrograde transport from endosomes to the Golgi apparatus. *Mol Biol Cell.* 2013;24(16):2570–81.
52. Pennauer M et al. Shared and specific functions of Arfs 1–5 at the Golgi revealed by systematic knockouts. *J Cell Biol,* 2022. 221(1).
53. Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol.* 1991;82(4):239–59.
54. Wang M, et al. Integrative network analysis of nineteen brain regions identifies molecular signatures and networks underlying selective regional vulnerability to Alzheimer's disease. *Genome Med.* 2016;8(1):104.

Figures

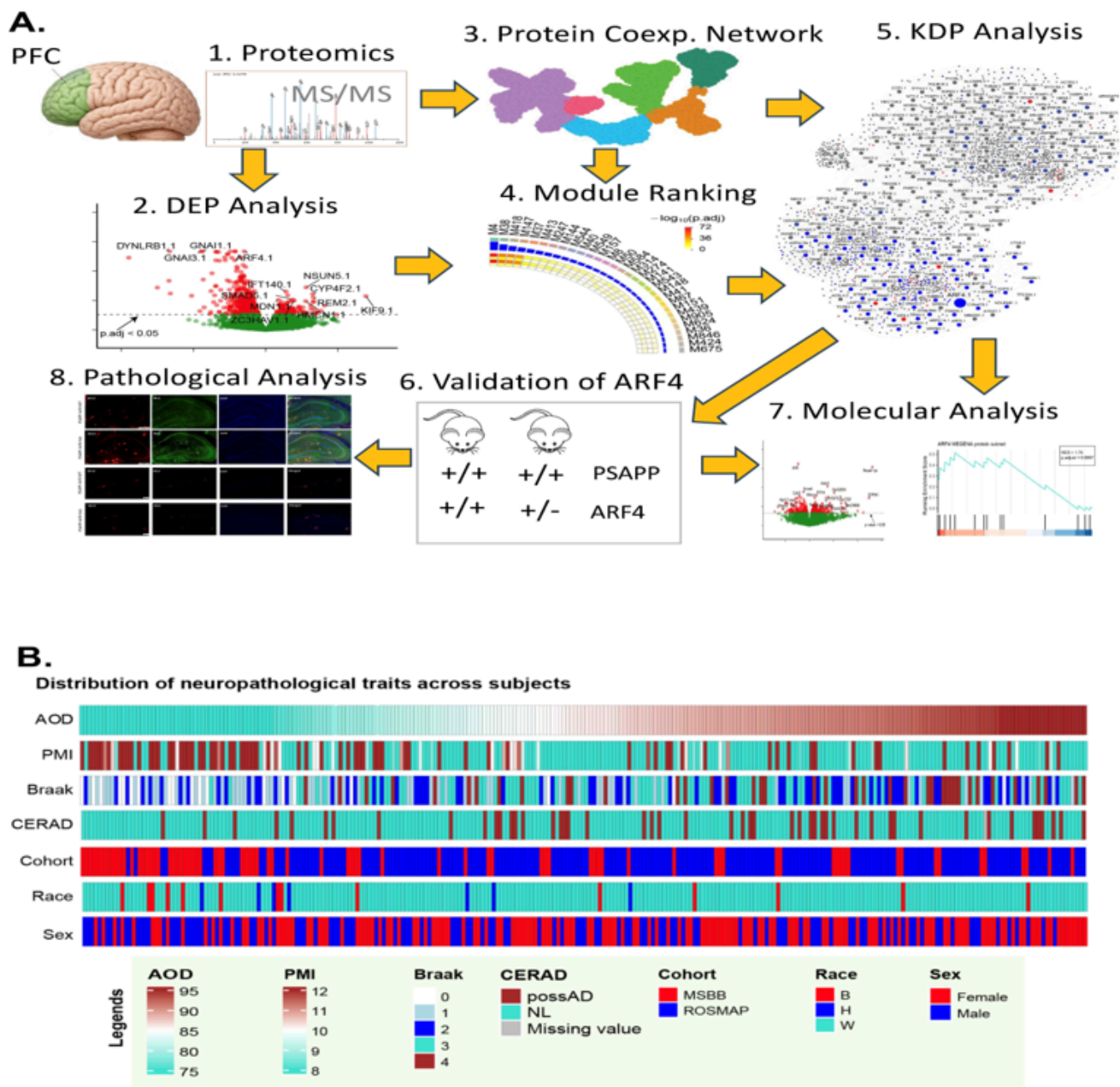


Figure 1

Figure 1

Outline of the study strategy and description of the neuropathological and clinical traits of human subjects. **A.** Outline of data generation, bioinformatic analysis, and animal experiments. The proteomic data first went through differential expression protein (DEP) analysis to identify the protein signature of OO and then the protein coexpression network analysis to identify OO associated protein modules. Meanwhile, key driver proteins of OO were also identified by integrating DEP and protein coexpression network analysis as well as the know AD risk genes. The top key driver of OO, ARF4, was evaluated in AD mouse models. Pathological and molecular analyses of ARF4 perturbations in PSAPP mice revealed the functions of ARF4 in neurodegeneration. **B.** Summary of the subject metadata. AOD, age at death. PMI,

post-mortem interval (hours). CERAD, the Consortium to Establish a Registry for Alzheimer's Disease. Braak, Braak stage score [53].

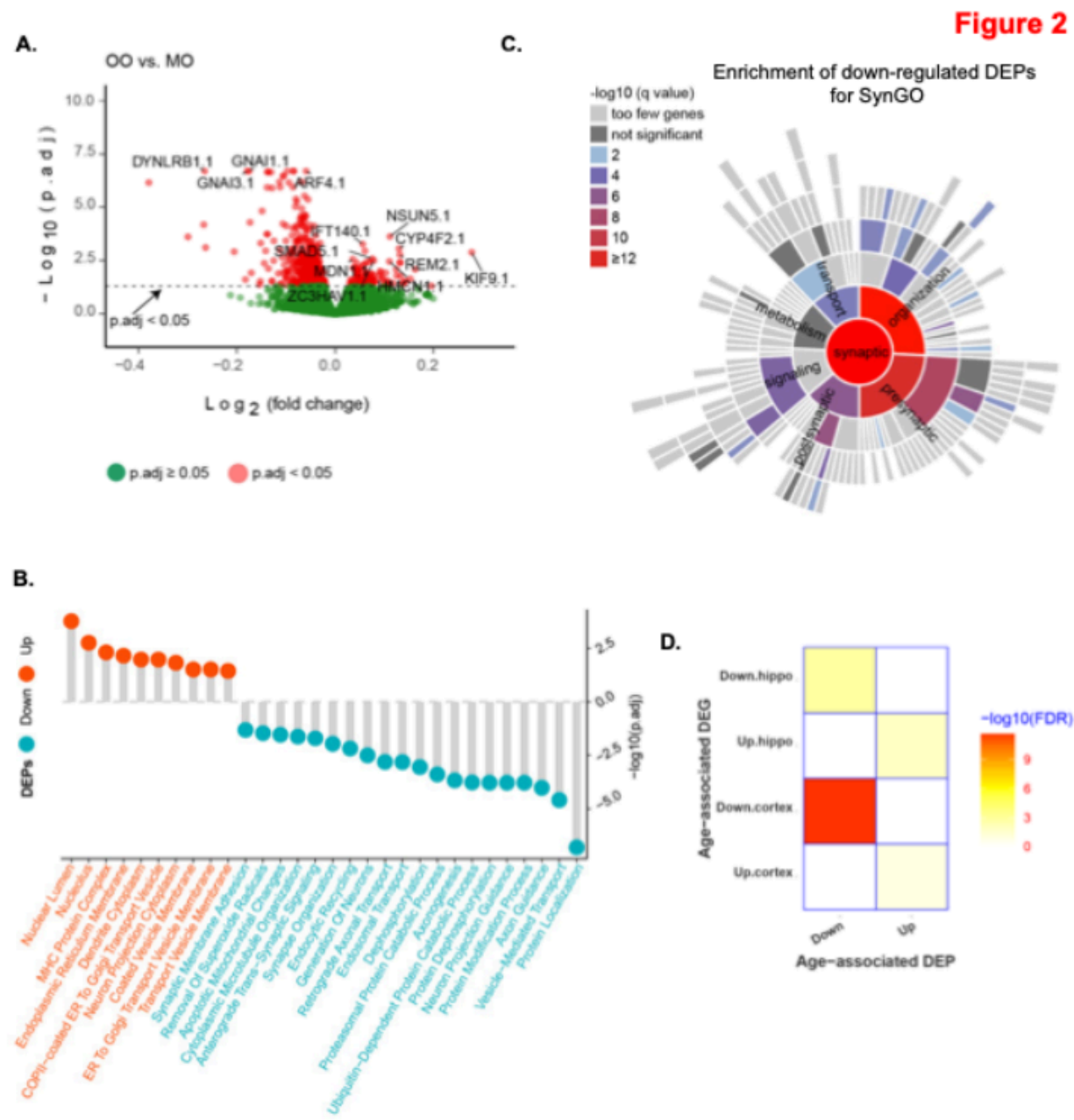


Figure 2

Analysis and integration of age-associated proteins. **A.** Volcano plot to show the differentially expressed proteins in the prefrontal cortex region of human healthy subjects in OO vs. MO stratified by age. OO, oldest old; MO, medium old. Note, each dot represents a distinct protein, and highlighted are the top-ranked DEPs with the lowest p.adj. The number after ‘.’ in a gene symbol denotes different protein isoforms. Red and green dots represent the DEPs and non-DEPs, respectively. Horizontal line denotes the cutoff adjusted p value < 0.05 . **B.** dot chart to display the enrichment of the DEPs for gene ontology (GO) terms. Down and up represent for down- and up-regulated in OO vs. MO. x-axis, various GO terms. y-

axis, $-\log_{10}(p.\text{adj})$ for enrichment for each GO term. **C.** the sunburst plot to show the synaptic localization of proteins that are downregulated in OO vs. MO. Highlighted are the first hierarchical layer of SynGO[40]terms. **D,** concordance of the DEPs over age-associated differentially expressed genes (DEG) derived from RNA-seq on human hippocampus or cortex. $p.\text{adj}$, adjusted p value.

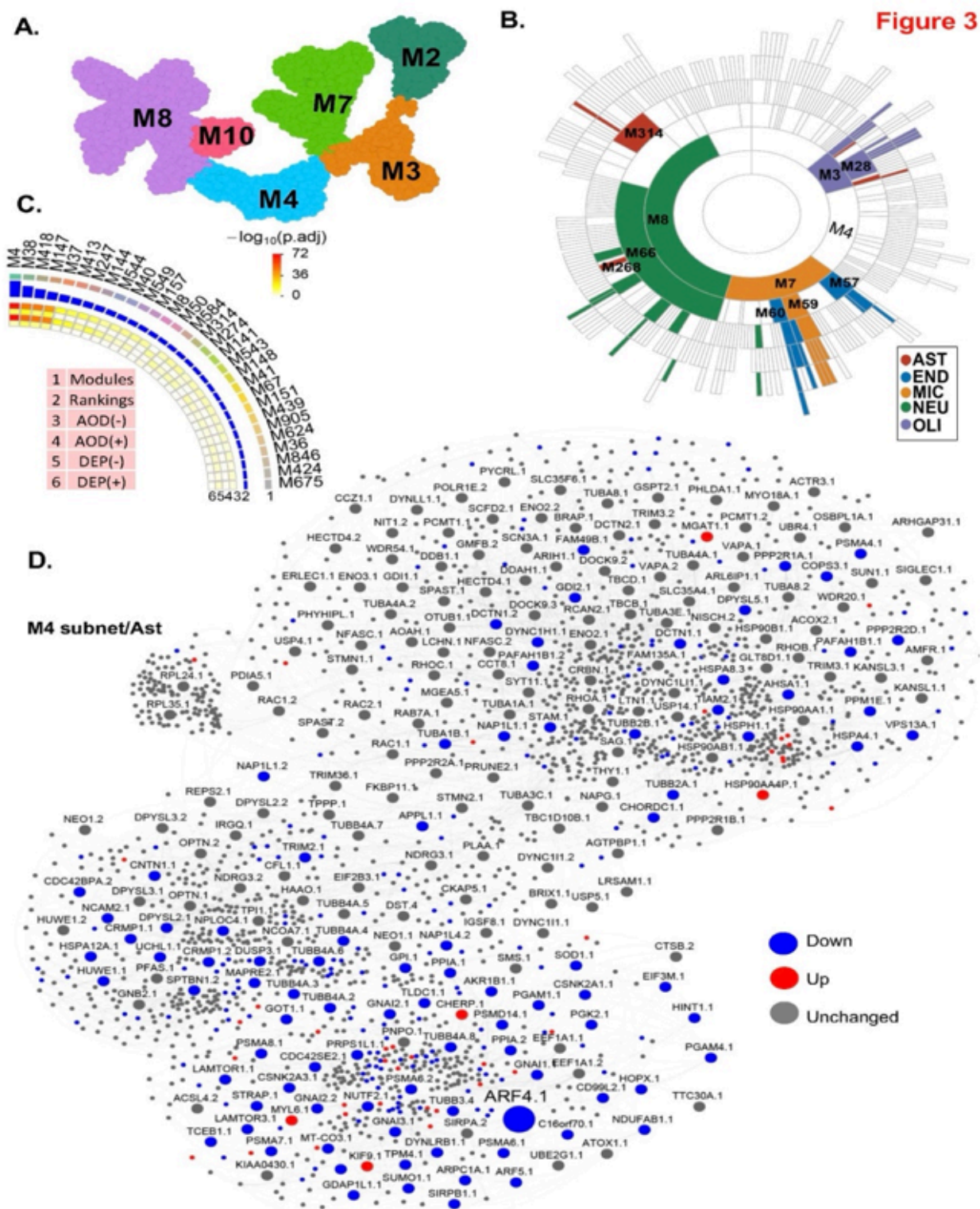


Figure 3

The integrative co-expression MEGENA network analysis of the proteome in the PFC of healthy human brains. **A.** Global MEGENA network with the modules of the first hierarchical layer highlighted. **B.** Module enrichment with the human brain cell-type markers[39]. In color are all the modules that are enriched for each human brain cell-type ($p_{\text{adj}} < 0.05$). Highlighted are the modules with the lowest enrichment adjusted p value per cell-type. **C.** circos plot to show the 30 top-ranked modules in relevance to age. Track 1 represents the top 30 modules. Track 2 denotes the rank order for the 30 modules. The modules are ranked according to the ensemble ranking metric[54] derived by summarizing the adjusted p values of the negatively- and positively-correlated protein signature (tracks 3 and 4), and the down- and up-regulated differentially expressed proteins (DEPs) (tracks 5 and 6). The height of each bar in blue is proportional to the inverse of the ranking order for the corresponding module, thus, the higher the bar the higher the module rank order. Tracks 3 through 6 are the heatmap to show the $-\log_{10}(p_{\text{adj}})$ value of the enrichment of each module for each of the age-correlated or DEP signatures. The legend is the $-\log_{10}(p_{\text{adj}})$: the higher the value the more enriched of the module for the age-associated protein signatures. p_{adj} , adjusted p value. **D.** The visualization of the MEGENA subnetwork M4. Each node represents an individual protein. Node colors indicate the change in expression in OO vs. MO. Red, up-regulated; blue, down-regulated; and gray, unchanged. The node size and label of key driver proteins are enlarged.

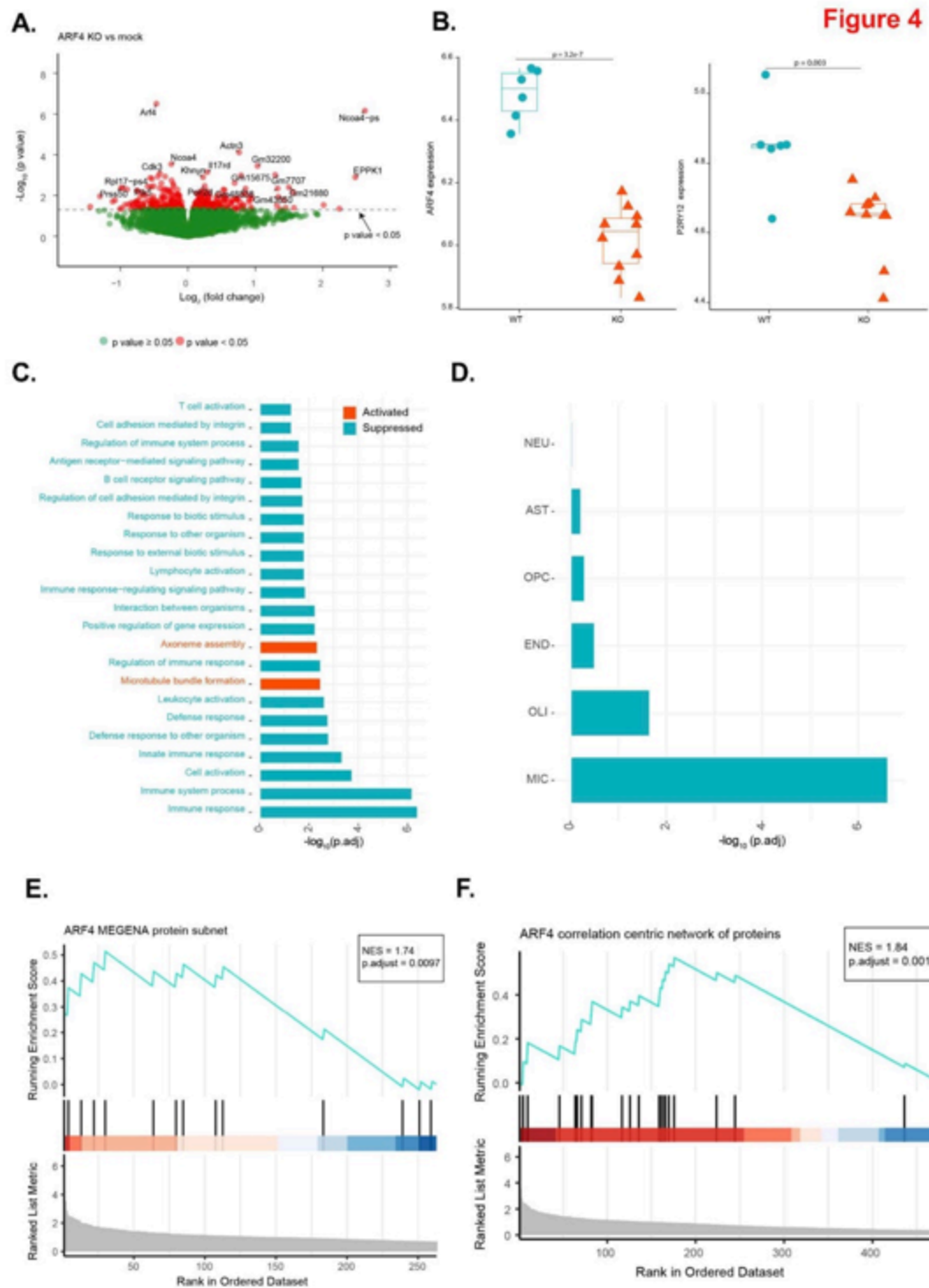


Figure 4

ARF4-knockdown in mice caused suppression of immune activities. **A.** Volcano plot showing the differentially expressed genes in the hippocampus region of PSAPP;Arf4(KO) vs PSAPP;Arf4(WT). Note, each dot represents a distinct gene, and highlighted are the top-ranked genes with the lowest p value. Horizontal line denotes the cutoff p value < 0.05. **B.** Boxplots to show the change in expression level for *Arf4* (left panel) and *P2ry12* gene (right panel) in PSAPP;Arf4(KO) vs. PSAPP;Arf4(WT) mice. **C.** Bar plot

to display the gene set enrichment analysis of *Arf4*-impacted genes for gene ontology (GO) terms. y-axis, the GO terms (biological processes) that are significantly enriched. Red and light blue shows activated- and suppressed-GO terms, respectively. x-axis, the $-\log_{10} (p.adj)$. **D.** Bar plot to display the gene set enrichment analysis of *Arf4*-impacted genes for mouse brain cell type gene signatures. y-axis, mouse brain cell types. Ast, astrocyte; Endo, endothelial cells; Mic, microglia; Neu, neurons; oligodendrocytes; Opc, oligodendrocyte precursor cells. x-axis, the $-\log_{10} (p.adj)$. **E.** Gene set enrichment analysis (GSEA) plot to show the enrichment outcome of the mouse *Arf4*-impacted gene signature in the ARF4 neighborhood in the protein MEGENA co-expression network. **F.** Gene set enrichment analysis (GSEA) plot to show the outcome of the mouse *Arf4*-impacted gene signature in the ARF4-correlated protein centric network. p.adj, adjusted p value.

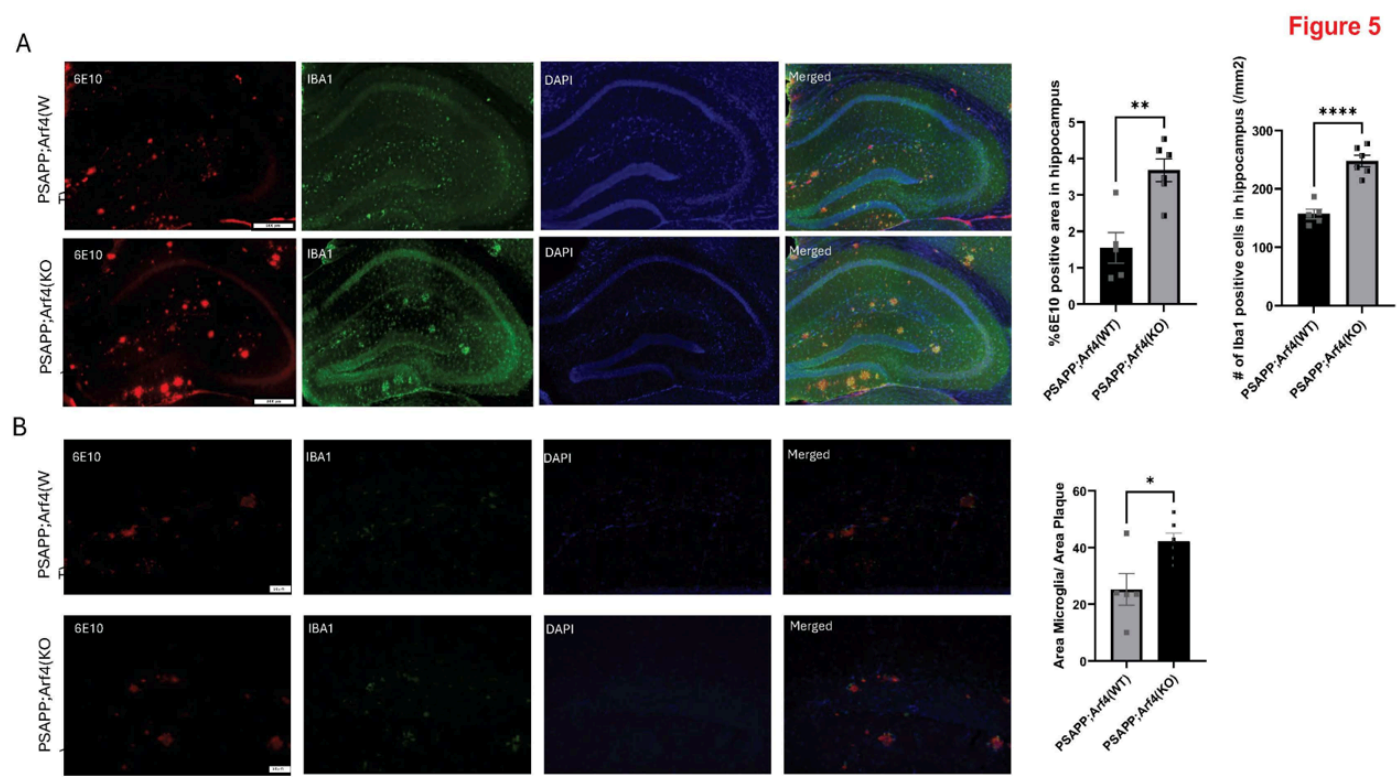


Figure 5

Increased AD pathology inPSAPP;*Arf4*(KO) mice brain. Representative images from 9-month-old PSAPP;*Arf4*(WT) and PSAPP;*Arf4*(KO) mice stained with 6E10 (amyloid, red), anti-IBA1 (microglia, green), and DAPI (Scale bar = 500 μ m). The percentage of 6E10-immunoreactive area in the hippocampi of female PSAPP;*Arf4*(WT) and PSAPP;*Arf4*(KO) mice (n = 5-6 mice per group) is shown. Quantification of the number of IBA1+ microglia in the hippocampus of the same sections is included. Quantification of plaque coverage by microglia (% area of IBA1 staining overlapping with 6E10+ plaques) for PSAPP-CO and PSAPP-TAM is shown (n = 5-6 mice per group) (Scale bar = 50 μ m). Bar graphs represent the mean,

and error bars indicate the standard error of the mean. p -values were calculated using an unpaired t-test and depicted as *p < .05, **p < .01, ***p < .001, ****p < .0001.

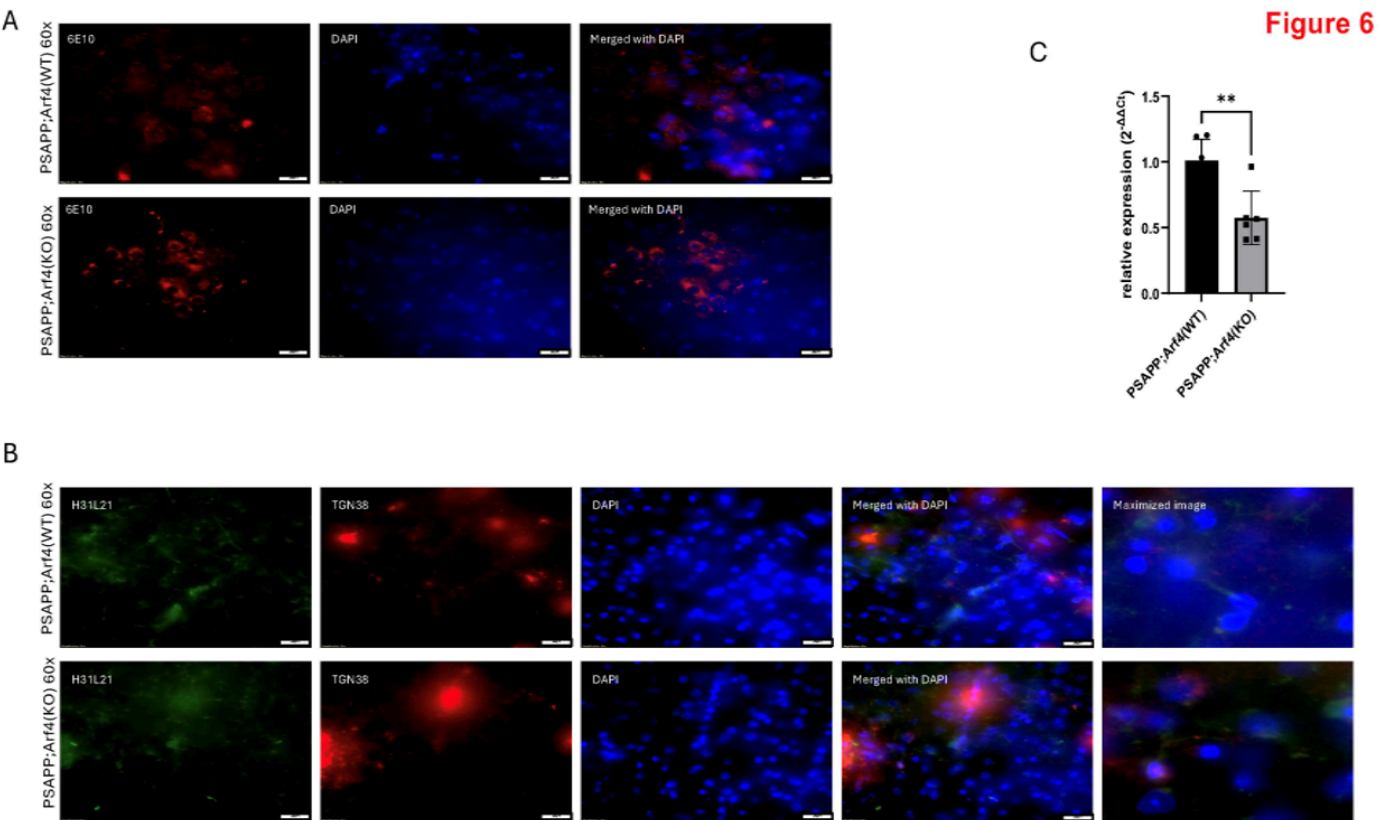


Figure 6

APP localization is altered by ARF4 knockdown. **A.** Localization of APP in high-power field images of 6E10-stained sections (60x, Scale bar = 20 μ m). **B.** Co-staining of sections with H31L21 (anti-Ab, red) and anti-TGN-38 (trans Golgi network, green) is presented (Scale bar = 20 μ m).

