

Subcellular proteomics and iPSC modeling uncover reversible mechanisms of axonal pathology in Alzheimer's disease

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1 SUPPLEMENTARY FIGURES

A. Human brain samples (frontal cortex) from Yale ADRC.

Brain Bank	AD/Control	ID	Age (years)	Sex	PMI (h)	AD neuropathological change	AD neuropathological level	Application
Yale ADRC	AD	40-0074	58	female	19	A2, B3, C3	intermediate AD	proteomics, IF
Yale ADRC	AD	40-0036	96	female	7	A2, B3, C2	intermediate AD	proteomics, IF
Yale ADRC	AD	400028	82	male	20	A3, B3, C3	high AD	proteomics, IF
Yale ADRC	AD	400126	75	female	3	A3, B3, C3	high AD	IF
Yale ADRC	AD	400032	84	male	22	A2, B3, C3	intermediate AD	IF
Yale ADRC	AD	400082	70	male	23	A2, B2, C3	intermediate AD	IF
Yale ADRC	AD	400192	83	female	19	A3, B3, C3	high AD	IF
Yale ADRC	AD	400098	83	female	17	A2, B3, C2	intermediate AD	IF
Yale ADRC	Control	400116	83	male	22	A0, B1, C0	NOT AD	proteomics, IF
Yale ADRC	Control	400070	84	male	27	A0, B1, C0	NOT AD	IF

B. Human brain samples (frontal cortex) from Banner Institute.

Brain Bank	AD/Control	ID	Age (years)	Sex	PMI (h)	PlaqueTotal	Plaque density	Braak score	NIA-R	AD neuropathological level	Application
Banner	AD	11-35	93	female	2.83	15	frequent	V	high	intermediate to high AD	IF
Banner	AD	12-27	89	female	2.7	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	12-49	63	male	5.17	14.5	frequent	VI	high	intermediate to high AD	IF
Banner	AD	13-42	80	male	3	14	frequent	VI	high	intermediate to high AD	IF
Banner	AD	13-54	85	female	3.42	14	frequent	VI	high	intermediate to high AD	IF
Banner	AD	13-75	77	male	3.62	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	14-48	73	male	4.83	14.5	frequent	VI	high	intermediate to high AD	proteomics, IF
Banner	AD	17-60	82	female	4	15	frequent	VI	high	intermediate to high AD	proteomics, IF
Banner	AD	12-49	63	male	5.17	14.5	frequent	VI	high	intermediate to high AD	proteomics, IF
Banner	AD	07-15	80	male	3.16	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	07-54	82	female	4	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	09-42	85	male	3	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	09-46	60	female	5	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	10-16	68	female	4.16	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	15-27	84	female	3.33	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	18-44	87	male	2.16	15	frequent	VI	high	intermediate to high AD	IF
Banner	AD	16-18	91	male	2.97	14.5	frequent	VI	high	intermediate to high AD	IF
Banner	Control	02-33	85	male	2.25	8.25	moderate	III	criteria not met	Not AD	IF
Banner	Control	04-52	85	male	1.83	6.25	sparse	II	criteria not met	Not AD	IF
Banner	Control	05-12	88	female	2	6.25	moderate	III	criteria not met	Not AD	IF
Banner	Control	98-22	90	male	2.5	8	moderate	III	criteria not met	Not AD	IF
Banner	Control	99-58	> 90	female	2.5	1	sparse	III	criteria not met	Not AD	IF
Banner	Control	18-17	87	female	3.08	0	zero	III	criteria not met	Not AD	proteomics
Banner	Control	18-78	71	male	3.5	0	zero	III	Criteria not met	Not AD	proteomics
Banner	Control	18-64	72	male	4.6	0	zero	I	Criteria not met	Not AD	proteomics
Banner	Control	15-60	82	male	3.33	0	zero	II	Criteria not met	Not AD	proteomics
Banner	Control	13-49	75	female	2.5	0	zero	II	criteria not met	Not AD	proteomics
Banner	Control	15-46	82	female	2.07	1	sparse	I	Criteria not met	Not AD	proteomics
Banner	Control	17-50	90	male	3.41	0.5	sparse	I	Criteria not met	Not AD	proteomics

C

Amyloid plaque characterization by ThioflavinS staining

Human AD

Unaffected control

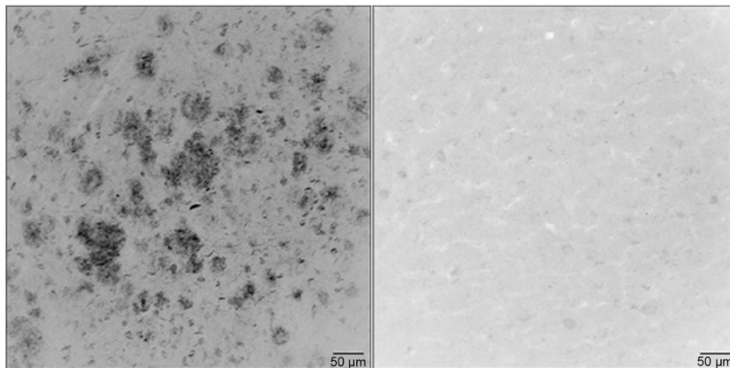
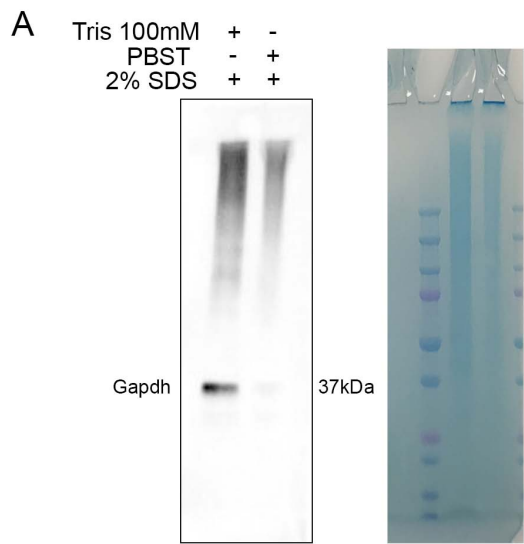


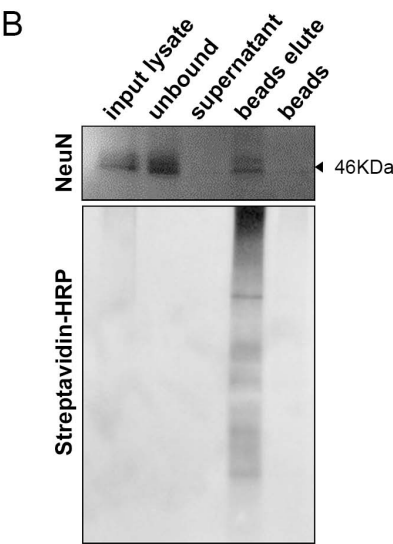
Figure S1. Metadata associated with postmortem human brains

A-B. Specimens obtained from: **(A)** the Yale Alzheimer's Research Center and **(B)** the Banner Sun Health Research Institute. IF = immunofluorescence validation. **C.** Representative large field of view images showing amyloid plaques (dark, ThioflavinS) in postmortem brains of AD humans and unaffected controls. Scale bar = 50 µm.

Optimization of protein extraction from fixed brain tissues



Pull-down of NeuN labeled proteins



Uncut images of western blots

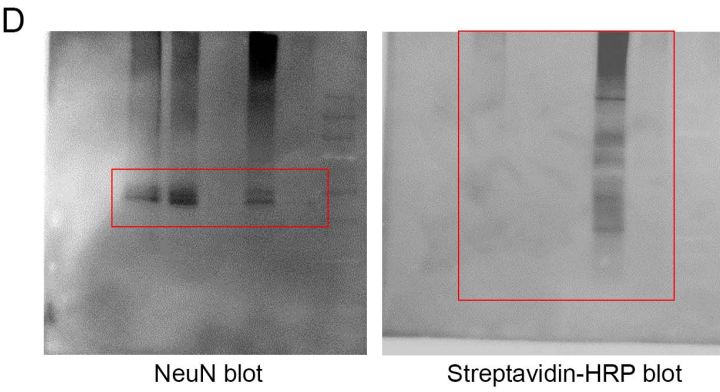
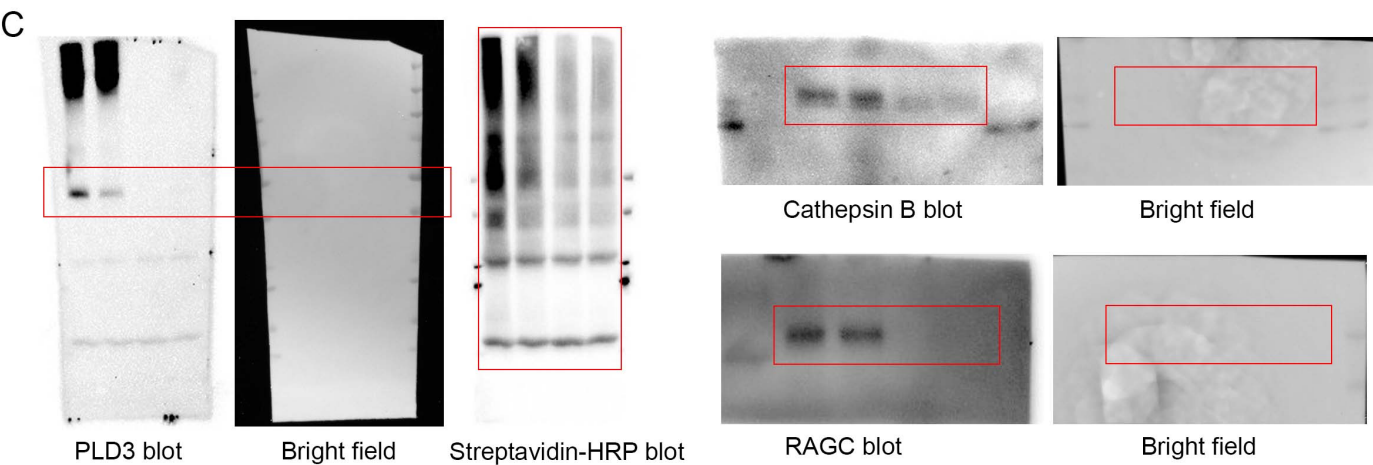


Figure S2. Optimization of protein extraction and western blots

A. Optimization of the protein lysis protocol for fixed postmortem brains. A lysis buffer containing 100 mM Tris-HCL and 2% SDS extracted proteins more efficiently than the previously published method using PBST with 2% SDS, as evidenced by a western blot for housekeeping protein GAPDH and a Coomassie blue-stained gel of the same membrane. **B.** Western blot of biotinylated proteins pulled down from an anti-NeuN antibody proximity-labeled sample. Biotinylated proteins, including the bait protein NeuN, were detected in input brain lysates and streptavidin bead elutes but not in the supernatant from bead washes or in the beads after elution. While bait proteins were detected in unbound protein fractions, no streptavidin signal was found in these fractions, indicating that there was a portion of endogenous protein baits that was not biotinylated, yet most of the biotinylated proteins were pulled down efficiently. **C-D.** Uncropped western blot membranes of **(C)** PLD3, Cathepsin B and RAGC pulled down, related to **Figure 1I**, and **(D)** NeuN pulled down, related to panel **(B)**.

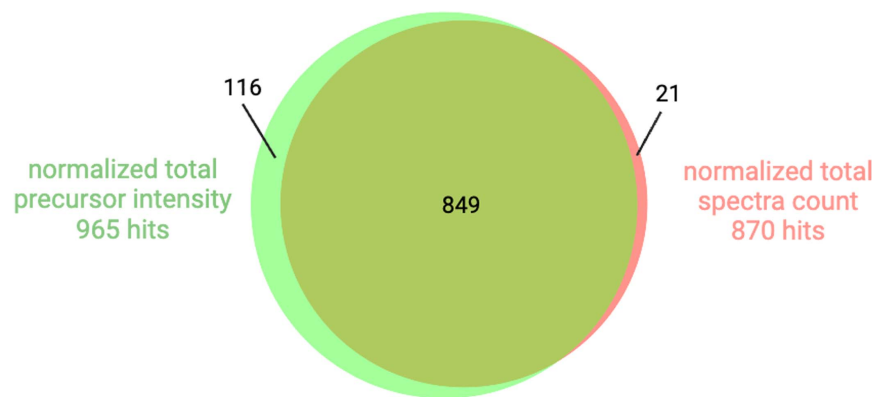


Figure S3. Comparison of quantification approaches in human PAAS proteomics.

Human PAAS proteomics analysis was performed using either normalized total precursor intensity (NTPI) or normalized total spectra count (NTSC) approaches. A Venn diagram showed the comparison of 965 proteomic (NTPI) hits and 870 proteomic hits (NTSC), highlighting their overlap.

A

Breakdown of proteins identified in PLD3-labeled and no antibody-labeled samples in AD humans.

	PLD3 antibody labeled						No antibody labeled					
	AD 1	AD 2	AD 3	AD 4	AD 5	AD 6	AD 1	AD 2	AD 3	AD 4	AD 5	AD 6
Proteins identified (2360 total)	1635	1526	1510	1127	1316	1075	765	809	909	1058	878	943

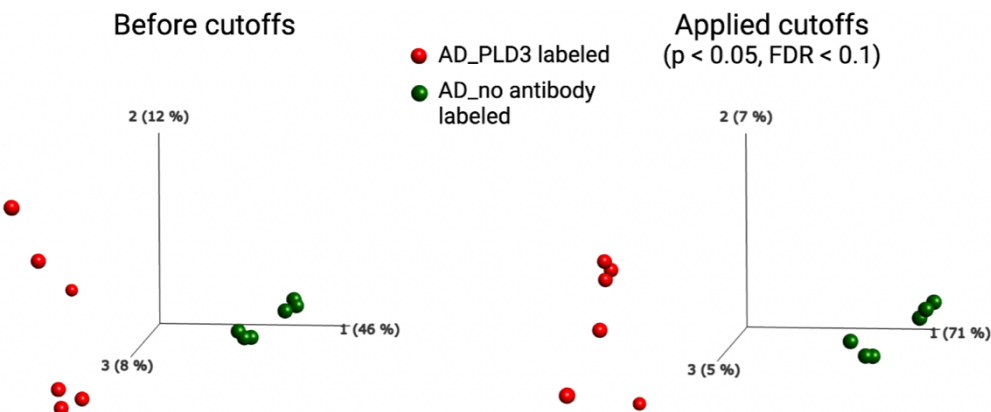
B

Breakdown of the PAAS proteomic hits identified in each AD human samples.

	AD human#1	AD human#2	AD human#3	AD human#4	AD human#5	AD human#6
Proteins identified (821 total)	793	801	800	722	765	676
Percentage of coverage	96.60%	97.56%	97.44%	87.94%	93.18%	82.34%

Figure S4. Breakdown of protein numbers identified in human samples.

A. Table displaying all proteins identified in PLD3-labeled and no-antibody controls for each AD human sample. **B.** Table showing the number of identified PAAS proteomic hits for each AD human sample.

**Figure S5. Principal component analysis (PCA) of PLD3-labeled versus no-antibody proteomes in AD human postmortem brains.**

PCA plots demonstrate that protein identification and quantification depend on the presence of the PLD3 antibody. Without applying cutoffs, PLD3 antibody-labeled samples exhibited clear separation from the no antibody-labeled samples. Furthermore, applying cutoffs ($p < 0.05$ and $FDR < 0.1$) further increased PC1 from 46% to 71%, enhancing differentiation. Quantification was performed two-sided.

List of known and newly validated proteins in PAAS of humans and mice

Protein	known/ newly validated PAAS proteins	identified in the PAAS proteomes	reference
PLD3	known	yes	Nackenoff et al, Plos Genet. 2021, Yuan et al, Nature, 2022
Cathepsin B (CTSB)	known	yes	Gowrishankar et al, PNAS, 2015;
Cathepsin D (CTSD)	known	yes	Gowrishankar et al, PNAS, 2015;
Lamp1	known	yes	Gowrishankar et al, PNAS, 2015;
neurofilament (NEFL, NEFM, NEFH)	known	yes	Gowrishankar et al, PNAS, 2015;
RagC (RRAGC)	known	yes	Gowrishankar et al, PNAS, 2015;
ATG9A	known	yes	Sharoar et al, Mol Psychiatry, 2019
Ubiquitin	known	yes	Sharoar et al, Mol Psychiatry, 2019; Torres et al, Mol. Neurodegener. 2012
Rtn1	known	yes	Shi et al, Sci Report, 2017
Rtn3	known	yes	Sharoar et al, Mol Psychiatry, 2019
PKC (alpha, beta, gamma) (PRKCA, PRKCB, PRKCG)	known	yes	Masliyah et al, J. Neurosci, 1991
Synaptophysin	known	yes	Adalbert et al, Brain, 2009
SNAP25	known	yes	Ferer et al, J. Neuropathol. Exp. Neurol. 1998
VAMP2	known	yes	Gowrishankar et al, PNAS, 2015;
beta tubulin III	known	yes	Sadleir et al, Acta Neuropathol, 2016
APP	known	yes	Yuan et al, Neuron, 2016
Atp6v0a1	known	yes	Yuan et al, Nature, 2022
Tau (MAPT)	known	yes	He et al, Nat Med, 2018; Bassil et al, Nat Commun. 2021
Phospho-Tau Ser202, Thr205, AT8	known	N/A	He et al, Nat Med, 2018; Bassil et al, Nat Commun. 2021
Phospho-Tau S235	known	N/A	Bassil et al, Nat Commun. 2021
Phospho-Tau S396	known	N/A	Bassil et al, Nat Commun. 2021
Phospho-Tau S404	known	N/A	Bassil et al, Nat Commun. 2021
Saposin	known	no (did not pass statistical cutoff)	Sharoar et al, Mol Neurodegener, 2021
BACE (D10E5)	known	no	Sadleir et al, Acta Neuropathol, 2016
LC3b	known	no	Sharoar et al, Mol Psychiatry, 2019; Torres et al, Mol. Neurodegener. 2012
M6PR	known	no	Gowrishankar et al, PNAS, 2015;
GRN	known	no	Gowrishankar et al, PNAS, 2015;
Synaptotagmin-11 (SYT11)	newly validated	yes	this study
SPTBN1	newly validated	yes	this study
APOE	newly validated	yes	this study
HDLBP	newly validated	yes	this study
C3	newly validated	yes	this study
HEXB	newly validated	yes	this study
TMEM30A	newly validated	yes	this study
mTOR	newly validated	yes	this study
p-mTOR S2448	newly validated	N/A	this study
LAMTOR1	newly validated	yes	this study
AKT1	newly validated	yes	this study
RagA (RRAGA)	newly validated	yes	this study
PIK3R4	newly validated	no (did not pass statistical cutoff)	this study
RHEB	newly validated	no (did not pass statistical cutoff)	this study
RAPTOR	newly validated	no (did not pass statistical cutoff)	this study
ITM2B	newly validated	yes	this study
ATP2B3	newly validated	yes	this study
Synaptotagmin-1 (SYT1)	newly validated	yes	this study
Calmodulin (CALM1)	newly validated	yes	this study
CAMK2A	newly validated	yes	this study
NTRK2	newly validated	yes	this study
GAPDH	newly validated	yes	this study
G3BP2	newly validated	yes	this study
PKAα/β cat (PRKACA, PRKACB)	newly validated	yes	this study
GAA	newly validated	yes	this study
SV2A	newly validated	yes	this study

38 **Figure S6. Known and newly validated PAAS proteomic hits in humans and mice**

39 This figure provides a list of known PAAS proteins and newly validated PAAS proteomic hits
40 identified in human and mouse samples. Full details, including antibody information,
41 immunofluorescence staining information, and quantification, are available in
42 **Supplementary Table S2.**

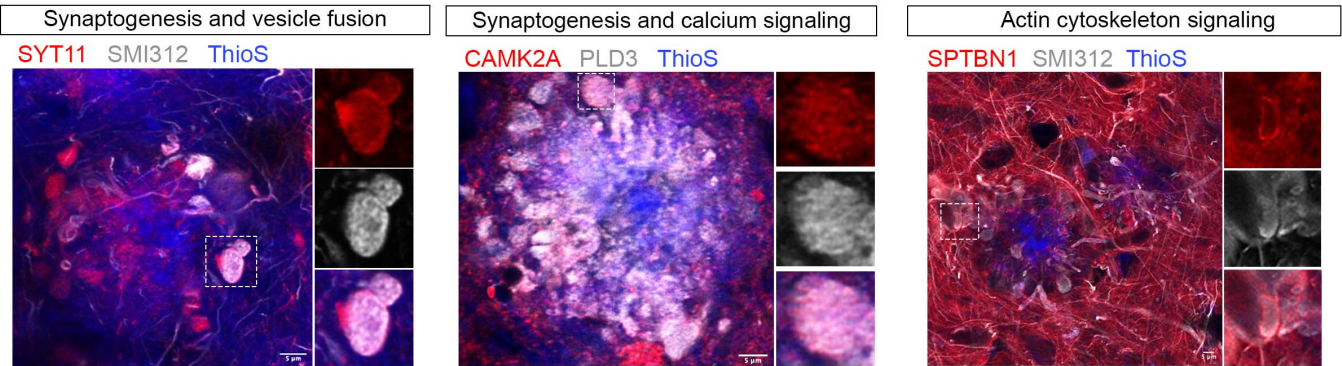


Figure S7. Immunofluorescence Validation of New Proteins in Axonal Spheroids in AD Human Postmortem Brains

Representative zoomed-out immunofluorescence confocal images show newly identified proteins related to synaptogenesis, vesicle fusion, calcium signaling, and actin cytoskeleton expressed in axonal spheroids in AD human postmortem brains (inserts shown in **Figure 3E**). Scale bar = 5 µm. Quantification was performed in n = 10 AD human brains. Protein expression data are provided in **Supplementary Table S2**.

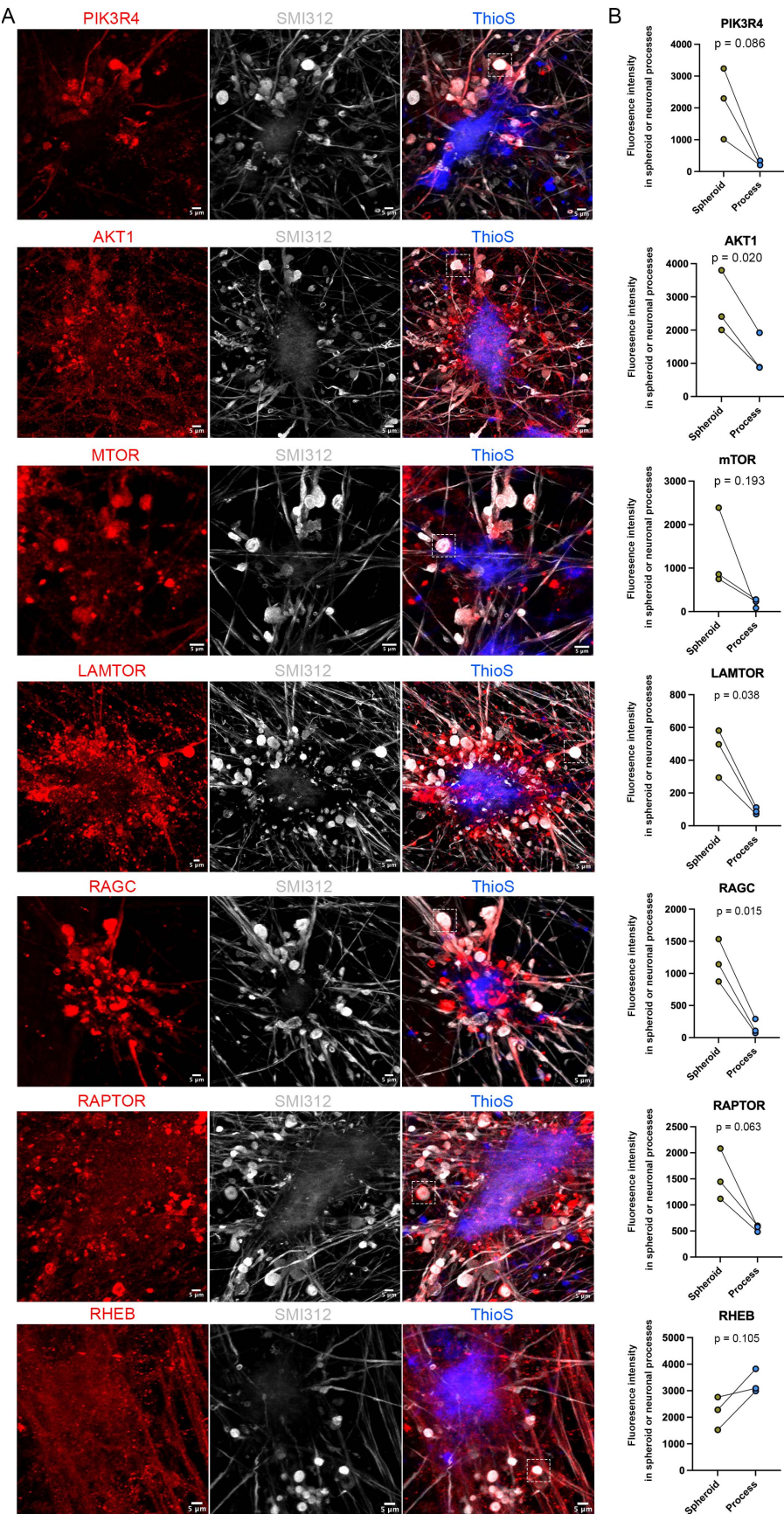
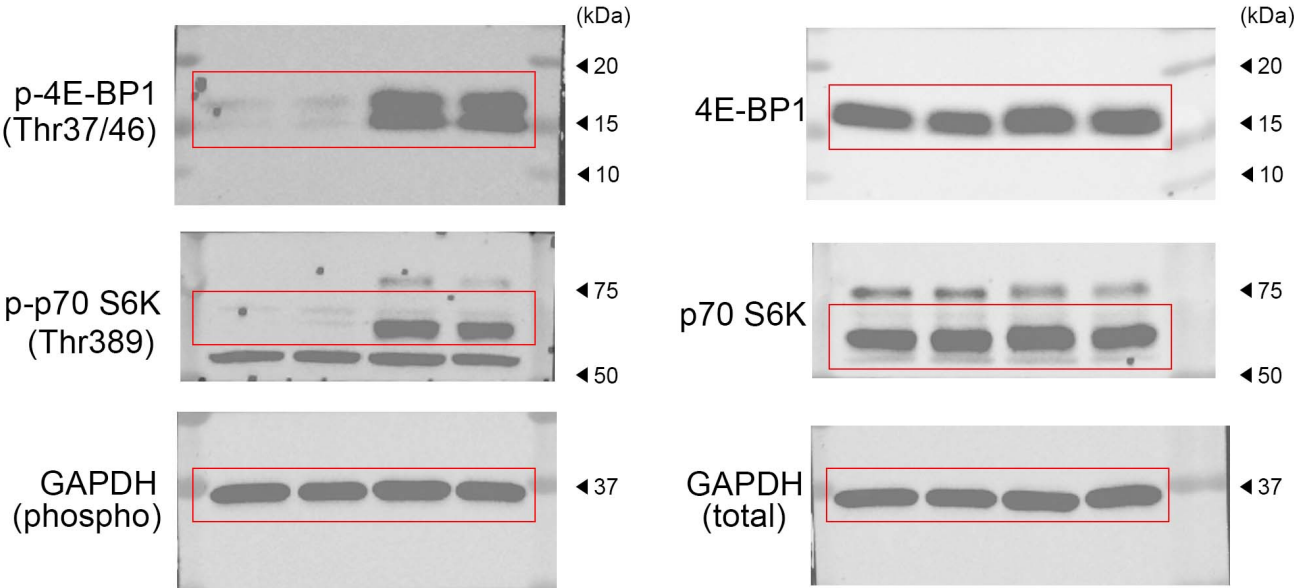


Figure S8. Immunofluorescence confocal imaging of the PI3K/AKT/mTOR signaling in human iPSC-derived neuron-astrocyte coculture AD model

A. PI3K/AKT/mTOR signaling molecules (red) are expressed in hiPSC-derived axonal spheroids (grey, SMI312) around amyloid plaques (blue, ThioflavinS). Cropped images of spheroids in white boxes from each large field-of-view image are shown in **Figure 6I**. Scale bar = 5 μ m. **B.** Quantification of fluorescence intensity of protein hits in axonal spheroids compared to neuronal processes. Dots represent three independent experiments. Paired t test was performed. Quantification was performed two-sided.

Figure S9. Western blot images of mTOR downstream substrates

Uncut images of western blots



Uncropped western blot membranes of mTOR downstream substrates, related to **Figure 6J**.

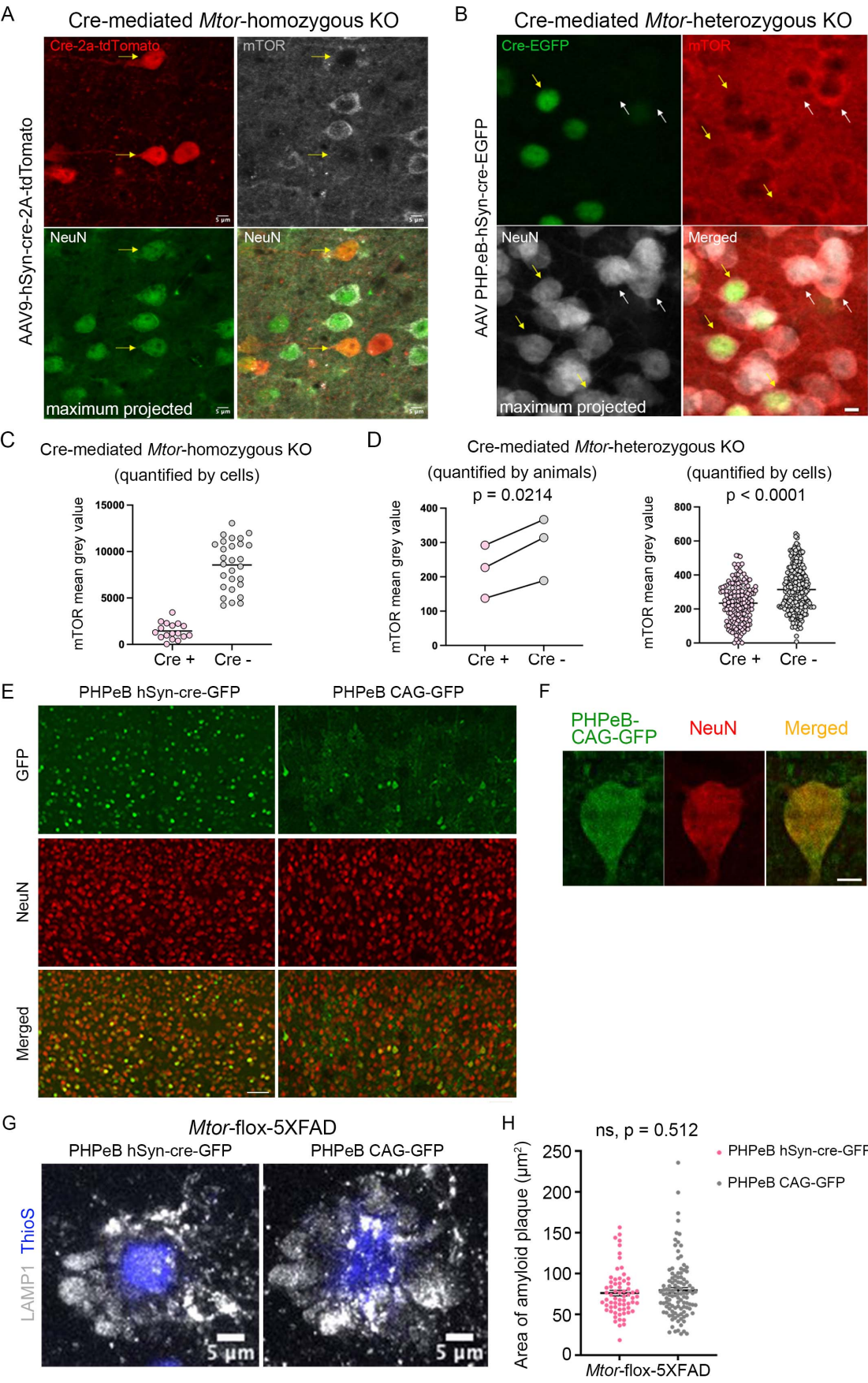


Figure S10. Genetic manipulation of mTOR signaling in mice

A. Uncropped immunofluorescence confocal images (shown in **Figures 7B and C**) demonstrate AAV9-hSyn-cre-2A-tdTomato-mediated complete *Mtor* knockout in homozygous *Mtor*-flox-5XFAD mice. mTOR expression was absent in Cre expressing neurons, compared to adjacent NeuN labeled neurons without Cre expression. **B.** AAV PHPeB-hSyn-cre-EGFP-mediated heterozygous mTOR knockout in heterozygous *Mtor*-flox-5XFAD mice. mTOR expression was reduced in Cre expressing neurons compared to adjacent NeuN-labeled neurons without Cre expression. **(A-B)** Scale bar = 5 μ m. **C-D.** Quantification of mTOR fluorescence intensity (mean grey value) shows both (C) homozygous and (D) heterozygous mTOR knockout significantly reduced mTOR expression. Cre-virus positive (knockout) cells were compared to Cre-virus negative (not knockout) cells in the same field of view. Cell bodies were outlined by NeuN staining. Homozygous *Mtor*-flox mouse (n=1), heterozygous *Mtor*-flox mice (n=3). **E.** Immunofluorescence confocal images showing AAV PHPeB hSyn-Cre-GFP virus infection efficiency in the somatosensory cortex of 5XFAD mice. Anti-NeuN staining indicates neuronal cell bodies. Scale bar = 50 μ m. **F.** Immunofluorescence confocal image showing NeuN-positive neuronal soma (AAV PHPeB-CAG-EGFP infected), used for soma size quantification in **Figure 7K**. Scale bar = 5 μ m. **G.** Representative images of axonal spheroid halos in heterozygous *Mtor*-flox-5XFAD mice under PHPeB-hSyn-cre-GFP (knockout) or PHPeB-CAG-GFP (not knockout) virus transduction. Spheroid halos were stained by LAMP1 (grey) and amyloid plaques were stained by ThioflavinS (blue). **H.** Quantification of individual amyloid plaque size within each ROI. Each dot represents a single amyloid plaque. *Mtor* knockout group: n = 66 plaques; control group: n = 109 plaques from 3 mice in each group. Unpaired t-test, two-tailed, p = 0.512. **(D and H)** Data are presented as mean values +/- SEM.

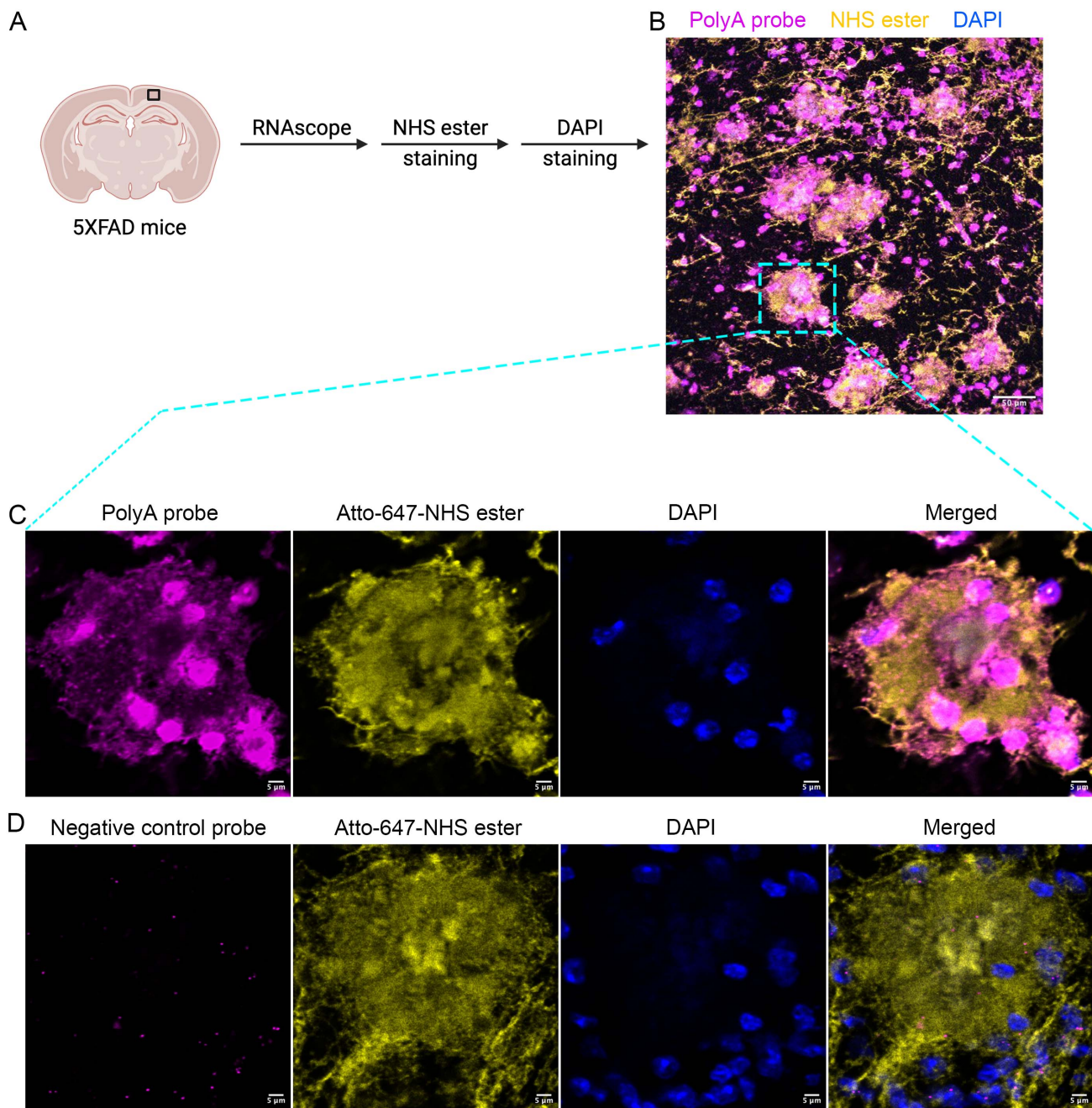


Figure S11. RNAscope revealed mRNA localization in axonal spheroids in 5XFAD mice

A. Schematic illustrating the RNAscope and staining pipeline applied to brain sections from 5XFAD mice. **B-C.** **(B)** Low-zoom and **(C)** high-zoom (inset, blue box) RNAscope images showing mRNA species (PolyA probe, magenta) highly expressed in cell bodies (DAPI, blue) and present within axonal spheroid halos (NHS ester, yellow). NHS ester labels axonal spheroid halos (including amyloid plaques), blood vessels and neuropil in the cortex of 5XFAD mice. **D.** High-zoom image of RNAscope using a negative control probe in 5XFAD mouse cortex. The RNA probe signal was largely absent in both cell bodies (DAPI, blue) and axonal spheroid halos (NHS ester, yellow). Scale bar **(B)** 50 μm , **(C-D)** 5 μm .

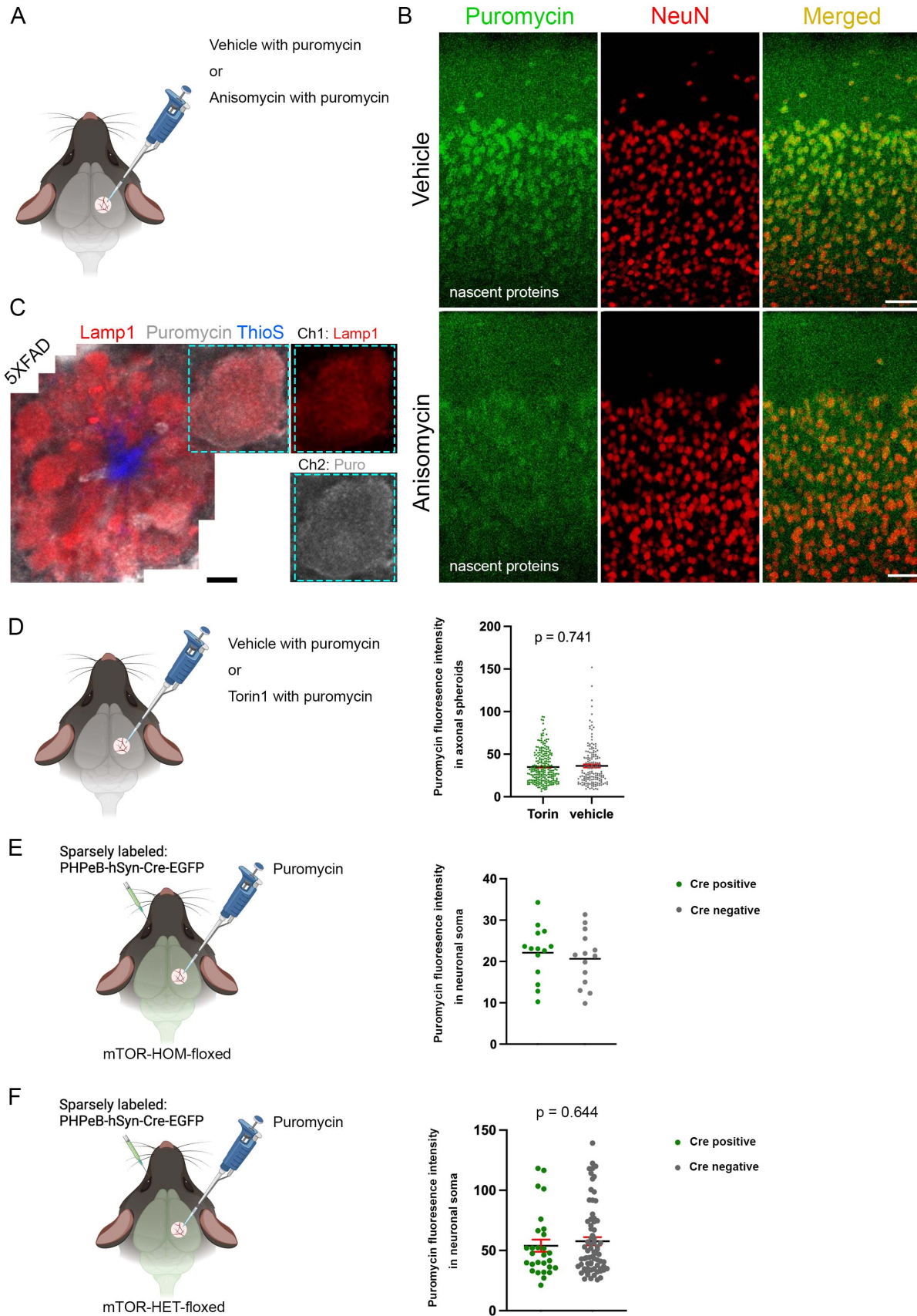


Figure S12. Puromycylation reveals nascent peptides in axonal spheroids in 5XFAD mice

A. Schematic illustrating puromycylation or anisomycin inhibition in mouse cortex. **B.** Immunofluorescence confocal images showing nascent proteins (puromycin-labeled) in the mouse cortex. Neuronal soma were labeled by NeuN. Anisomycin inhibition reduced the degree of puromycin labeling. **C.** Representative images showing nascent proteins (puromycin-labeled, grey) in axonal spheroids (Lamp1-labeled) surrounding amyloid plaques (ThioflavinS-labeled, blue) in 5XFAD mice. Scale bars = 5 μ m. **D.** Quantification showing that Torin1 (700 μ M) treatment for two hours did not affect puromycin labeling in axonal spheroids. Each dot represents an axonal spheroid, from n = 3 mice. **E-F.** Schematics of **(E)** homozygous knockout and **(F)** heterozygous mTOR knockout achieved by transducing PHP.eB-hSyn-Cre-GFP viruses in the cortex of homozygous or heterozygous mTOR- floxed mice. Quantification showed that neither homozygous **(E)** nor heterozygous **(F)** mTOR knockout impacted puromycin labeling in neuronal soma. Each dot represents a Cre-positive, or Cre-negative NeuN-labeled cell body) from **(E)** mTOR-HOM-floxed mouse (n = 1), and **(F)** mTOR-HET-floxed mice (n = 4). Unpaired t-test, two-tailed was performed. **(D, F)** Data are presented as mean values +/- SEM.

SUPPLEMENTARY TABLES

Table S1: Raw and analyzed proteomics results for PAAS, Lamp1-proximity labeling and neuronal soma proteomics in humans and mice.

Table S2: Lists of newly identified and known PAAS proteins, along with antibody details immunofluorescence staining instructions.

Table S3: Proteomic sample information (also available at ProteomeXchange).

Table S4: Pathway enrichment analysis of Gene Ontology (GO) terms for PAAS proteomes in AD humans and mice.

Table S5: IPA signaling pathway analysis of PAAS proteomes in AD humans and mice.

Table S6: GSEA analysis of PLD3-labeled proteomes in AD humans versus unaffected controls.

SUPPLEMENTARY MOVIE CAPTION

Movie S1: A 3D video captured using FIB-SEM from a 12-month-old 5XFAD mouse showing spheroids (magenta) filled with a large number of vesicles, located around amyloid plaques (cyan).