

1 Quantitative phosphoproteomics 2 uncovers dysregulated kinase 3 networks in Alzheimer's disease

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16 Abstract

17 Alzheimer's disease (AD) is a form of dementia characterized by amyloid- β plaques and Tau
18 neurofibrillary tangles that progressively disrupt neural circuits in the brain. The signaling networks
19 underlying the pathological changes in AD are poorly characterized at the level phosphoproteome. Using
20 mass spectrometry, we performed a combined analysis of the tyrosine, serine, and threonine
21 phosphoproteome, and proteome of temporal cortex tissue from AD patients and aged matched
22 controls. We identified several co-correlated peptide modules that were associated with varying levels
23 of phospho-Tau, oligodendrocyte, astrocyte, microglia, and neuronal pathologies in AD patients. We
24 observed phosphorylation sites on kinases targeting Tau as well as other novel signaling factors that
25 were correlated with these peptide modules. Finally, we used a data-driven statistical modeling
26 approach to identify individual peptides and co-correlated signaling networks that were predictive of AD
27 histopathologies. Together, these results build a map of pathology-associated phosphorylation signaling
28 events occurring in AD.

29 Introduction

30 Alzheimer's disease (AD) is a neurodegenerative disease commonly associated with aging. AD
31 has an immense disease burden, with an estimated 50 million cases worldwide and a total economic
32 cost of more than \$500 billion per year¹. Despite numerous clinical trials, a limited understanding of
33 disease pathogenesis has hindered the development of effective therapies. Amyloid beta (A β) plaques
34 have been identified as a hallmark pathology of AD that develops early on, followed by accumulation of
35 hyper-phosphorylated microtubule-associated protein Tau (MAPT) and eventual neurodegeneration².

36 Throughout AD, cellular signaling through protein phosphorylation events mediates A β and MAPT
37 production and cytotoxic mechanisms as well as other stress-responses³. Previous work has identified
38 GSK3 β , CDK5, Protein Kinase A, CaMKII, Fyn, and members of the BRSK, MARK, MAPK, and Casein Kinase
39 families as enzymes that can phosphorylate MAPT and promote its aggregation⁴⁻⁷. Other dysregulated
40 kinases include PKC α as a mediator of synaptic defects^{8,9}, JAK as an activator of inflammation^{10,11}, and
41 mTOR as a regulator of neuronal homeostatic adaption¹². Targeting some of these kinases has shown
42 positive effects in AD models^{8,13,14}, suggesting that modulating cellular signaling through protein
43 phosphorylation may be a viable treatment strategy for AD. However, there have been few studies
44 mapping these signaling events to stages of AD progression using knowledge of the phosphoproteome.

45 Tools to measure the proteome and phosphoproteome have been developed using mass
46 spectrometry (MS)¹⁵⁻²⁰. Previous studies have analyzed the phosphoproteome of AD mouse models²¹⁻²³
47 as well as the global phosphoserine/phosphothreonine (pSer/pThr) phosphoproteome and global
48 proteome of post-mortem AD brain tissue^{21,24-30}. However, these studies were limited by an incomplete
49 characterization of the phosphoproteome that did not include critical phosphotyrosine (pTyr) sites that
50 mediate the activity of many known kinases. Many of these studies were also limited to a small number
51 of patients or pooled samples together, which hinders the development of a quantitative model of
52 disease progression. We aimed in this study to integrate pTyr, deep pSer/pThr phosphoproteome, and
53 deep proteome measurements of AD and age-matched brain tissues over a range of disease stages. Our
54 data collection strategy allowed us to associate phosphoproteome changes with direct measurements of
55 MAPT phosphorylation, cell-type specific markers, and clinical histopathology measurements.

56 Co-correlation analysis of this dataset identified several clusters that were enriched for neuronal
57 and glial-specific proteins; these clusters were associated with Tau pathology, oligodendrocyte changes,
58 and neurodegeneration. Our analysis recaptured the links between Tau pathology and several known
59 MAPT kinases and highlighted kinases and signaling factors that were previously unreported to be
60 associated with AD. These factors include creatine kinase B-type (CKB), cyclin-dependent kinases (CDKs)
61 CDKL5, CDK16, CDK17, and the tyrosine kinases SRC, DDR1, EGFR, and PTK2. Integrative analysis of
62 phosphoproteomic and proteomic data with patient histopathology measurements highlight intriguing
63 association between many of these signaling factors and AD pathologies, providing new insight into
64 disease progression. Together these data underscore the value of phosphoproteomics technologies in
65 building a quantitative understanding of disease etiology at a systems-scale.

66 Methods

67 Human brain samples

68 Samples of human middle temporal gyrus (MTG) were secured from AD or neurologically
69 normal, non-demented (ND) elderly control brains obtained from rapid autopsy at the Banner Sun
70 Health Research Institute Tissue Bank (BSHRI)^{31,32}. Cognitive status of all cases was evaluated
71 antemortem by board-certified neurologists, and postmortem examination by a board-certified
72 neuropathologist resulting in a consensus diagnosis using standard NIH AD Center criteria for AD or ND.
73 The AD and ND groups were well matched for age, sex and PMI. ND control cases: ND Braak I n=5, ND
74 Braak IV n=18 (23 total); Age: 84.1 \pm 7.1 years; Gender: 12 females and 11 males; Postmortem interval
75 (PMI: 2 hours 58 minutes +/- 75 min. AD cases: AD Braak IV n=10, AD Braak V n=8, AD Braak VI n=7; Age:
76 84.3 +/- 7.9 years; Gender: 11 females and 14 males; PMI: 3 hours 23 min +/- 180 min. ND patients

77 were classified as APP⁺ if they had a Plaque density value that was not “zero”. The full list of each ID, sex,
78 age, and diagnostic histopathology measurements for patients is provided in **Supplementary Table 1**.

79 Sample Processing for Proteomics

80 MS-based analysis of pTyr, pSer/pThr, and protein expression were conducted using a standard
81 analysis pipeline developed in the White lab and used in numerous studies and publications^{19,33-36}. 20 -
82 60 mg of frozen tissues for proteomics analysis were transferred into 3 mL ice-cold 8M Urea and
83 immediately homogenized to denature proteins and preserve physiological signaling. Lysates were then
84 centrifuged at 4000xg for 30 minutes to clear lysates of lipids and then assayed by BCA to determine
85 protein concentration. Lysates were then treated with 10 mM DTT for 1 hour at 56°C, followed by 55
86 mM iodoacetamide for 1 hour at room temperature, rotating in the dark. Samples were diluted by
87 adding 8 mL Ammonium Acetate pH 8.9 and then digested at room temperature overnight on a rotator
88 with trypsin at a 1:50 ratio of trypsin to total protein mass. This reaction was quenched with 1 mL
89 99.99% Acetic Acid and samples were desalted using Sep-Pak Plus cartridges. Peptides were then dried
90 to half volume in a speed-vac and post-cleanup concentrations were determined using a colorimetric
91 peptide assay. Peptides were then divided into 130 µg aliquots that were snap-frozen and lyophilized.
92 Peptides were then labeled with 10-plex isobaric Tandem Mass Tags (TMT) using a reduced volume
93 protocol³⁷. Labeled peptide samples were then dried down in a speed-vac overnight.

94 Peptide Fractionation and Phosphopeptide Enrichment

95 Phospho-enrichment experimental design is pictured in **Figure 1a**. Tyrosine phosphorylated
96 peptides were enriched by a two-step process consisting of an immunoprecipitation (IP) with multiple
97 pan-specific anti-phosphotyrosine antibodies (4G10, PT66) followed by immobilized metal affinity
98 chromatography (IMAC) using hand-packed micro-columns as we have previously described^{33,38-41} as
99 as well as with commercial IMAC spin-columns. IP supernatants were subjected to a second round of IP
100 enrichment with anti-MAPK-CDK phospho substrate antibody followed by IMAC cleanup using
101 commercial spin-columns. Small amounts of each IP supernatant were diluted in 0.1% acetic acid for
102 global protein expression profiling. IP supernatants were then divided across 80 fractions using high pH
103 reverse phase chromatography on a ZORBAX C18 column. Fractions were concatenated into 20 tubes
104 and dried down. Each fraction was then enriched using commercial Fe-NTA columns. Small amounts of
105 each fraction were diluted in 0.1% acetic acid for deep proteome profiling.

106 For commercial spin-columns, Thermo High-Select Fe-NTA columns were washed twice with 200
107 µL Binding/Wash Buffer and beads were resuspended in 25 µL Binding/Wash Buffer. Peptides were
108 eluted from IP beads using two rounds of 10 min washes with 25 µL 0.2% trifluoroacetic acid (TFA) using
109 the same pipette tip to transfer eluates directly onto Fe-NTA beads. Phosphopeptides were incubated
110 with beads for 30 minutes with gentle tapping. Beads were then washed twice with 200 µL
111 Binding/Wash Buffer, and once with 200 µL LC-MS water. Phosphopeptides were eluted into BSA-coated
112 microcentrifuge tubes using two 20 µL washes of Phosphopeptide Elution Buffer. Samples were brought
113 down to 1-5 µL volume by speed-vac for ~20 minutes and then 10 µL 0.1% acetic acid with 2%
114 acetonitrile was added. Samples were loaded directly onto a hand-packed, BSA-conditioned 10 cm
115 analytical column with 5 µm C18 beads. Columns were rinsed with 0.1% acetic acid to remove excess
116 salts and then analyzed by liquid chromatography-tandem mass spec (LC-MS/MS) on QExactive HF-X
117 Orbitrap mass spectrometer.

118 For LC analysis, Agilent 1100 Series HPLCs were operated at 0.2 mL/min flow rates with a pre-
119 column split to attain nanoliter flow rates through the analytical column and nano-ESI tip. Peptides were
120 eluted with increasing concentrations of buffer B (70% acetonitrile, 0.1% acetic acid) using the gradient
121 settings: 0-13% (10 min), 13-42% (95 min), 42-60% (10 min), 60-100% (7 min), 100% hold (6 min), 100-
122 0% (2 min). Global phosphoproteome and proteome fractions were analyzed using an EASY-nLC nano-
123 flow UHPLC. Fractions were eluted using the gradient settings: 0-10% (10 min), 10-30% (100 min), 30-
124 40% (14 min), 40-60% (5 min), 60-100% (2 min), hold 100% (10 min), 100-0% 2 min. Peptides were
125 ionized by electrospray ionization (ESI) at 1 - 3 kV.

126 Peptides were analyzed by LC-MS/MS on a QExactive Plus and QExactive HF-X Orbitrap mass
127 spectrometer operating in data-dependent mode acquiring MS scans and HCD MS/MS fragmentation
128 spectra. Ions with charge >1 were dynamically selected for fragmentation using a top-20 untargeted
129 method with an exclusion time of 30s and ion isolation window of 0.4 Da. The maximum injection time
130 and ACG targets were set to 50 ms and 3e6 respectively. MS scans were captured with resolution =
131 60,000 and MS2 scans with resolution = 45,000. Peptides were fragmented with the HCD normalized
132 collision energy set to 33%. Protein expression profiling was performed on LTQ Orbitrap or QExactive
133 Plus instruments.

134 Data Processing and Analysis

135 Peptide identification and quantification was performed using Proteome Discoverer and
136 MASCOT. Raw files were searched against the 'SwissProt_2020_02.fasta' for tryptic peptides from *Homo*
137 *sapiens* with ≤2 missed cleavages. Precursor and fragment ions were matched with a 10 ppm and 20
138 mmu mass tolerances respectively. Variable modifications were included for phosphorylation (Ser, Thr,
139 Tyr), oxidation (Met), and TMT 10plex (Lys, N-term) and fix modifications were set for carbamidomethyl
140 (Cys). False discovery rates for peptide-spectrum matches (PSMs) were estimated using the Percolator
141 module and post-translational modification (PTM) localizations were calculated using ptmRS⁴². ptmRS
142 operated with a peak depth of 15. For pTyr analyses, a diagnostic ion of 216.04 with a peak depth of 15
143 was used. For MAPK/CDK substrate, pSer/pThr, and proteome analyses, a neutral loss of 98 (-H₃PO₄) was
144 included for pSer/pThr ions.

145 Searched .msf files were then imported into Python and processed using pyproteome
146 (<https://github.com/white-lab/pyproteome>). PTMs were localized using a ptmRS confidence cutoff of
147 0.75. PSMs were filtered for ion score ≥15, isolation interference ≤30, median TMT signal ≥1500, and
148 Percolator FDR ≤1e-2. TMT quantification data was then normalized using an iterative fitting procedure
149 based on the CONSTrained STANdardization algorithm⁴³. In this procedure, the matrix of filtered TMT
150 quantification values was iteratively adjusted such that rows were mean-centered around 1 and
151 columns were mode-centered around 1. Mode centers were calculated by fitting a gaussian KDE
152 distribution (`scipy.stats.kde.gaussian_kde`) to column intensities and finding the max value of each
153 probability density function. Duplicate PSMs were then grouped for final quantification using a weighted
154 mean function: $TMT * (1 - \frac{\text{isolation interference}}{100})$ for the TMT intensities and isolation interference value
155 quantified by Proteome Discoverer for each PSM. Final relative TMT intensities were normalized to ND
156 APP control samples across TMT 10-plex analyses. Data clustering, statistical analysis, and figure
157 generation was performed using *sci-kit learn*⁴⁴, *scipy*⁴⁵, *numpy*⁴⁶, *pandas*⁴⁷, *seaborn*⁴⁸, and *matplotlib*⁴⁹.

158 Clustering and regression analysis

159 Clustering analysis was performed on the unique peptide abundance matrix using rows with no
160 missing values for peptides that matched ≤ 4 proteins. Mini-Batch K-means clustering was performed
161 using scikit-learn's `MiniBatchKMeans` function. A cluster number of 100 was selected based on an
162 analysis of cluster membership properties. Cluster centroids were calculated from the mean \log_2 value
163 of all peptides assigned to a cluster.

164 For partial least squares regression (PLSR) analysis, the X matrix was generated from all cluster
165 centroid \log_2 values and the Y matrix was generated from histopathology measurements. Select
166 histopathology measurements were translated into discrete numbers that were then z-scored. 2-
167 component PLSR models were generated using pypls, a Python implementation of the SIMPLS algorithm.
168 A K-fold cross validation procedure was run for each histopathology variable with splits=5 and
169 repeats=100. R^2 values were equal to the Spearman ρ^2 between the true and predicted Y vectors for the
170 training datasets. Q^2 values were similarly calculated from the true and predicted Y vectors from the test
171 datasets. A final 2-component PLSR model was calculated from non-redundant histopathology variables
172 with $Q^2 > .3$. PLSR model variables are related by the equation $X * X \text{ weights} = X \text{ scores}$ and $Y_{\text{approximate}} = Y$
173 $\text{scores}_{\text{truncated}} * Y \text{ loadings}_{\text{truncated}}$, where truncated indicates that the model is being truncated at 2 latent
174 variables.

175 Explained variance for Y matrices was calculated as the ratio of the sum of squares of the Y_{loadings}
176 matrix to the sum of squares of the Y matrix. A similar formula was used for the ratio of the X_{loadings}
177 matrix (calculated from the pseudoinverse of the X_{weights}) to the X matrix.

178 To select strongly-associated variables, Variable Importance in Projection (VIP) scores were
179 calculated using the previously-described formulae⁵⁰:

$$SS_a = \left(\sum_{k=1}^p (X_{\text{scores}_{k,a}})^2 \right) * \left(\sum_{k=1}^p (Y_{\text{loadings}_{k,a}})^2 \right)$$
$$VIP_k = \sqrt{\frac{p * \sum_{a=1}^h SS_a * \left(\frac{X_{\text{weights}_{k,a}}}{\|X_{\text{weights}_a}\|} \right)^2}{\sum_{a=1}^h SS_a}}$$

180 Where a is the ath component, k is the kth predictor variable, p is the number of predictor
181 variables, h is the number of components.

182 Correlation cutoffs were estimated by generating a null distribution from all peptides quantified
183 in ≥ 15 samples. For each cluster, we calculated Spearman's correlation between peptides and scrambled
184 cluster centroid values. We repeated this procedure 100 times and the 99th percentile value was
185 estimated from all calculated Spearman ρ and Spearman p-values. The final ρ and p-value cutoffs of 0.47
186 and 5e-3 were selected as cutoffs that matched less than 0.24% of all peptides for any of our six named
187 cluster centroids. Spearman partial correlation coefficients were calculated using
188 `pingouin.pairwise_corr`. Receiver operating characteristic (ROC) curves were generated using
189 `sklearn.metrics.roc_curve`.

190 Cell type enrichment and composition analysis

191 Gene cell-type predictions were estimated using a cell-type specific sequencing atlas^{51,52}. Cell
192 expression values were extracted from Table S4 using the following columns: 'Astrocyte': ['8yo', '13yo',
193 '16yo', '21yo.1', '22yo.1', '35yo', '47yo', '51yo', '53yo', '60yo', '63yo - 1', '63yo - 2'], 'Neuron': ['25 yo'],
194 'OPC': ['22yoGC', '63yoGC - 1', '63yo GC - 2', '47yoO4', '63yoO4'], 'New Oligodendrocytes': ['22yoGC',
195 '63yoGC - 1', '63yo GC - 2', '47yoO4', '63yoO4'], 'Myelinating Oligodendrocytes': ['22yoGC', '63yoGC - 1',
196 '63yo GC - 2', '47yoO4', '63yoO4'], 'Microglia': ['45yo', '51yo.1', '63yo'], 'Endothelia': ['13yo.1', '47yo.1'].
197 Genes were calculated as being enriched in a given cell type if they met the criteria $\frac{\mu_{cell\ type}}{\sum_{i \neq cell\ type} \mu_i} > 2.5$,
198 where μ is the mean FPKM value from collections of columns for each cell type. OPC and New
199 Oligodendrocytes were excluded from the enrichment calculation to avoid exclusion of pan-
200 oligodendrocyte genes, only 'Myelinating Oligodendrocytes' was used for the displayed
201 'Oligodendrocyte' category.

202 For cell type composition analysis, peptides were processed into a list of unique cell type-
203 enriched genes with at least one PSM (modified or unmodified) that was strongly correlated with each
204 centroid (Spearman $\rho > .7$; Spearman p-value $< 1e-3$; # patients ≥ 15). Phosphorylation composition was
205 performed similarly by mapping each unique peptide to 'pY' if it contained at ≥ 1 pTyr modification, 'pST'
206 if it contained ≥ 1 pST modification, or 'no phospho' if it was not phosphorylated. Cluster cell type and
207 phosphorylation significance was calculated using scipy's 'chi2_contingency' comparison between each
208 cluster centroid and the background dataset of peptides (# patients ≥ 15).

209 Kinome analysis

210 Kinase phosphopeptides that were quantified in ≥ 15 patients and were significantly correlated
211 with Tau or Oligo were used for kinome analysis. We generated vectors for each peptide that were
212 equal to the Spearman partial ρ between that peptide and each cluster centroid (ρ_{Tau} , ρ_{Oligo}), with the
213 alternative cluster centroid as the covariate. Correlation vectors were filtered to have angles between -
214 25° and 110° , corresponding to the range of kinase phosphosites that were significantly correlated with
215 Tau or Oligo clusters. Phosphopeptides mapping to more than one kinase are used to color all nodes. For
216 each kinase, the phosphopeptide with the largest vector magnitude was used to calculate node color
217 and size. Kinome maps were generated using Coral⁵³ with nodes and branches colored by the correlation
218 vector angle.

219 Binomial enrichment analysis

220 Binomial enrichment analysis of pTyr, pSer/pThr, and protein data subsets was performed using
221 gene sets derived from published proteomics data²⁶ and single-nuclei RNA-seq data⁵⁴. Peptides were
222 processed into a background collection of unique genes that were enriched in any gene set. Only
223 peptides that mapped to a single protein were used. Foreground collections were generated from
224 peptides that had spearman correlation $>.7$ with cluster centroids. Log odds enrichment (LOE) values
225 were calculated as $-\log_{10} \frac{sf(k-1)}{cdf(k)}$, with k = the count of genes in a given gene set from the foreground
226 collection. Survival functions (sf) and cumulative distribution functions (cdf) were calculated using
227 scipy's hypergeometric distribution model (scipy.stats.hypergeom), with: n = the total number of
228 foreground genes; K = the count of genes in the gene set; and N =the total number of background genes.

229 Phosphosite Enrichment Analysis

230 Phosphosite enrichment analysis (PSEA) were performed using the procedure outlined in a
231 previous publication⁵⁵. We used a custom phosphosite database derived from Phosphosite Plus (PSP)⁵⁶.
232 Kinase-substrate mappings were downloaded from PSP using information for all species
233 (Kinase_Substrate_Dataset.gz), and then re-mapped to human phosphosites using homology
234 information from PSP (Phosphorylation_site_dataset.gz). BRSK1 and BRSK2 phosphosite sets were
235 generated from a recent phosphoproteomics analysis⁵⁷ using the table of filtered and imputed
236 abundance values with a MaxQuant localization probability > 0.6, log₂ Fold Change of 1.5, and 2-sample
237 T-test p-value < 1e-2 for log₂ abundance values.

238 Phosphosite changes were estimated from the median value of redundant phosphopeptides.
239 Spearman correlations with cluster centroids were z-scored and phosphosites were rank ordered.
240 Enrichment scores were calculated using the integral of a running-sum statistic (exponential weight =
241 0.75) that was increased for each gene or site contained within a given set and decreased for each gene
242 not contained in that set. Enrichment scores for 1000 matrices with scrambled rows were generated and
243 used to calculate empirical p-values, q-values, and normalized enrichment scores (NES). Only
244 phosphosite sets with a minimum overlapping set size of 20 were scored.

245 Mouse Phosphoproteome Analysis

246 The phosphoproteome of AD mouse models was downloaded from a recent paper that was
247 collected using a similar TMT quantification and phosphopeptide enrichment strategy
248 (ProteomeXchange identifier: PXD018757)²². Mouse phosphosites were categorized as upregulated
249 (Fold Change > 1.25; p < 1e-2) or downregulated (Fold Change < .8; p < 1e-2) for all tissues and disease
250 timepoints in each model (CK-p25: 2wk Hippocampus + Cortex, 7wk Hip; 5XFAD: 9-10mo Hippocampus +
251 Cortex; Tau P301S: 4+6 mo Hippocampus). Phosphosites were mapped from mouse to human using the
252 most recent version of 'Phosphorylation_site_dataset.gz' from PSP⁵⁶. Missing phosphosite mappings
253 were automatically generated by matching a 9-mer around the mouse phosphosite to corresponding
254 human protein sequence using Python's `difflib.SequenceMatcher` function. Phosphosite overlap was
255 calculated between each mouse model and the human phosphosites that were correlated with each
256 cluster centroid (Spearman ρ > .47; Spearman p-value < 5e-3; # patients \geq 15). Phosphoproteins were
257 counted if they had at least one homologous phosphopeptide that was associated with a cluster.

258 Data and Code Availability

259 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
260 Consortium via the PRIDE⁵⁸ partner repository with the dataset identifier PXD020087 and
261 10.6019/PXD020087.

262 The proteomics data integration software is available on GitHub ([https://github.com/white-](https://github.com/white-lab/pyproteome)
263 [lab/pyproteome](https://github.com/white-lab/pyproteome)). This repository also includes detailed software for concatenating peptide fractions on
264 a Gibson FC 204 Fraction Collection (<https://github.com/white-lab/fc-cycle>), and an updated tool for
265 validating PSMs (<https://github.com/white-lab/CAMV>). Sources for other code used in this study are
266 indicated in the Resources Table.

267 Resources Table

Reagent	Source	Catalog Number / Version
Human brain samples of postmortem	Banner Sun Health	N/A

Alzheimer's disease and controls	Research Institute	
Urea	Sigma	Cat# U5128
Pierce BCA Protein Assay	Thermo Fisher Scientific	Cat# 23225
Dithiothreitol (DTT)	Sigma	Cat# D0632
Iodoacetamide (IAA)	Sigma	Cat# I1144
Sequencing Grade Modified Trypsin	Promega	Cat# V5111
99.99% Acetic Acid	Sigma	Cat# 338826
Sep-Pak Plus C18 Cartridge	Waters	Cat# WAT020515
Pierce Quantitative Colorimetric Peptide Assay	Thermo Fisher Scientific	Cat# 23275
Tandem Mass Tag 10-plex	Thermo Fisher Scientific	Cat# 90110
ZORBAX 300Extend-C18	Agilent	Cat# 770995-902
High-Select™ Fe-NTA Phosphopeptide Enrichment Kit	Thermo Fisher Scientific	Cat# A32992
10 μm C18 beads	YMC	Cat# ODS-A AA12S11
5 μm C18 beads	YMC	Cat# ODS-AQ AQ12S05
Fused Silica Capillary Tubing 50 μm ID	Polymicro Technologies	Cat# 1068150017
Fused Silica Capillary Tubing 100 μm ID	Polymicro Technologies	Cat# 1068150023
Fused Silica Capillary Tubing 200 μm ID	Polymicro Technologies	Cat# 1068150204
Anti-phospho-Tyr (4G10)	Millipore	Cat# 05-321; RRID:AB_568857
Anti-phospho-Tyr (PT66)	Sigma	Cat# P3300; RRID:AB_477335
Anti-MAPK/CDK Substrate (34B2)	Cell Signaling Technology	Cat# 2325; RRID:AB_331820
Q Exactive Plus	Thermo Fisher Scientific	IQLAAEGAAPFALGMBDK
Q Exactive HF-X	Thermo Fisher Scientific	0726042
Python	Python Software Foundation	3.8.3
Proteome Discoverer	Thermo Fisher Scientific	2.2.1
MASCOT	Matrix Science	2.4.1
pyproteome	PyPI	0.11.0
scikit-learn	PyPI	0.22.1
scipy	PyPI	1.4.1
matplotlib	PyPI	3.1.1
numpy	PyPI	1.18.1
pandas	PyPI	1.0.3
seaborn	PyPI	0.10.1
pingouin	PyPI	0.3.6
pyls	GitHub	0.18

268 Results

269 Proteomic and phosphoproteomic analyses identify molecular hallmarks of AD

270 In order to analyze the AD proteome and phosphoproteome, we generated proteolytic peptide
 271 digests of medial temporal gyrus (MTG) cortical brain tissue from patients with late-onset AD and age-
 272 matched non-diseased (ND) controls. Previous work has shown that the proteome of this region is
 273 affected by AD in a similar manner to other brain regions²⁶. We used isobaric tandem mass tags (TMT) to

274 label peptides, allowing us to perform multiplexed relative quantification of peptide abundances across
275 samples. Using a sample processing and peptide enrichment scheme outlined in **Figure 1a**, we
276 quantified relative changes in the proteome and phosphoproteome of 48 patient samples (AD n=25; ND
277 n=23; Age=70-90; Female=23; Male=25; **Supplementary Data 1**). We performed an analysis of the low-
278 abundance pTyr phosphoproteome as well as a deep fractionated analysis of pSer/pThr
279 phosphoproteome and proteome to generate a large dataset of peptide-spectrum matches (PSMs). By
280 integrating TMT abundances across all runs, we constructed a matrix of relative peptide abundances in
281 each patient that we could use to interrogate signaling network changes. This strategy led to the
282 identification and quantification of 88,419 unique peptides (1.5% pTyr, 16% pSer/pThr, **Supplementary**
283 **Data 2**), of which 58% of all peptides were identified in at least 15 patient samples (≥ 2 10-plex analyses)
284 (**Supplementary Figure 1a-d**). To assess the reproducibility of our quantification method, we compared
285 peptide abundances between technical replicates of TMT10-plex analyses. To assess biological
286 variability, we additionally analyzed two independent brain samples in separate TMT10-plex analyses for
287 two patients. We found that peptide abundances were well correlated between samples for both
288 technical and biological replicates (**Supplementary Figure 1e-h**). With a sensitive and quantitative data
289 analysis strategy, we next examined the molecular features within individual patient proteomes and
290 phosphoproteomes.

291 Our proteomics dataset successfully recapitulates hallmark AD pathologies, including a tryptic
292 peptide mapping to $A\beta_{17-28}$ (LVFFAEDVGSNK) that was upregulated in some ND patients and plateaued in
293 AD Braak IV-VI cases (**Figure 1c**). Additionally, we identified phosphopeptides from MAPT (KLDLSNVQsK:
294 pS606; IGsTENLK: pS579) that were increased in AD patients (**Figure 1d, 1e**). Within the pTyr dataset, we
295 observed increased phosphorylation of the non-receptor tyrosine kinase SRC (GAYcLSVSDFDNAK: pY187;
296 **Figure 1f**). We note that these peptides are upregulated at different Braak stages and have different
297 ranges of variability, reflecting the complex etiology of AD combined with other potential patient
298 comorbidities. Together, this dataset allows us to infer aspects of disease pathology and link those
299 measurements to the phosphosites regulating kinase activity in a given tissue sample.

300 Clustering analysis identifies Tau and cell-type peptide modules

301 In order to identify major trends of covariation in our dataset that may be reflective of different
302 types of pathology in each patient, we applied clustering analysis to extract co-correlated peptide
303 changes. We performed K-means clustering on the 8,706 peptides that were quantified in all 48 patients
304 (1.6% pTyr, 13% pSer/pThr; **Supplementary Data 3**). We selected a cutoff of n=100 clusters to balance
305 the intra-cluster correlation value with the number of redundant clusters (**Supplementary Figure 2a-2c**).
306 Of the 100 clusters, we manually assigned identities to six clusters that explained 73% of all cluster
307 variance. “Tau” is a cluster that includes several MAPT peptides that were upregulated in AD patients;
308 rank-ordering the patient samples based on the signal intensity of the Tau cluster effectively separates
309 the ND and AD patients, albeit with a few exceptions (**Figure 2a**). “Oligo”, “Astro”, and “Micro” are three
310 clusters containing proteins primarily expressed in oligodendrocytes (MBP, PLP1), astrocytes (GFAP),
311 and microglia (GNA13) respectively (**Figure 2b-d**). “Neuro” and “pNeuro” are two clusters containing
312 proteins (SYT1, SYN1, SNAP25) and phosphopeptides (CAMKK1, GSK3A, CASKIN1) involved in neuronal
313 synaptic signaling; both of these clusters were downregulated in subsets of AD patients (**Figure 2e, 2f**).
314 We examined the composition of predicted cell types for peptides that were strongly correlated with
315 each cluster’s centroid and found that the Oligo, Astro, Micro, and Neuro clusters were significantly
316 enriched for each respective cell type (**Figure 2g**). In addition, the Tau and pNeuro clusters contained an

317 increased fraction of pSer/pThr peptides compared to the full dataset (**Supplementary Figure 2d**). The
318 cluster centroids for Tau and Oligo were independent (correlation ≈ 0), while Astro and Micro were
319 correlated with one another as well as with Tau and Oligo (**Figure 2h**). In contrast, the Neuro cluster was
320 most strongly anti-correlated with Oligo and Micro (**Figure 2h**).

321 To confirm that these trends are not caused by tissue-sampling artifacts, we applied a series of
322 enrichment analyses using AD-specific knowledge bases. We first examined the overlap between our
323 dataset and a recently published large-scale proteomics analysis of AD patients²⁶. We find that the Oligo
324 protein modules in both datasets had a strong degree of overlap (**Supplementary Figure 2e**). In addition,
325 proteins from that study's Astro / Micro module were enriched in our Astro and Micro clusters, while
326 the Synapse / Neuron and Mitochondrial modules were enriched in our Neuro cluster. We next
327 calculated the overlap between peptides associated with each cluster and the marker genes for cell type
328 populations observed in a previous single-nuclei RNA-seq (snRNA-seq) analysis of cortical AD tissue⁵⁴.
329 We observed that a number of marker transcripts for the Oli0 (oligodendrocyte) population that was
330 associated with late-stage pathology (*e.g.* *QDPR*, *CRYAB*) were similarly upregulated at the level of
331 phosphorylation and total protein (**Supplementary Figure 2f**). In addition, we observed that the AD-
332 associated astrocyte (Ast1-3) and excitatory / inhibitory neuron (Ex2-8, In0-8) populations overlapped
333 with our Astro and Neuro clusters respectively. Interestingly, the snRNA-seq analysis did not detect
334 differential regulation of myelin proteins such as MBP, CNP, or PLP1 that were seen in both proteomics
335 analyses (**Supplementary Figure 2g, 2h**). Thus, we find that the cell type-specific changes that are seen
336 in our clusters overlap with previous AD proteomics and snRNA-seq analyses^{26,54}.

337 We next calculated the ability of our cluster centroids to estimate AD pathologies. Using a
338 receiver operating characteristic (ROC) curve analysis, we found that Tau was the best predictor of AD
339 status (**Figure 2i**), in agreement with the rank order separation of AD and ND cases by Tau cluster
340 centroid values (**Figure 2a**). We then projected samples into 2 dimensions using principal component
341 analysis (PCA) of the peptide matrix used for clustering. We find that AD and ND samples are separated
342 along principle component 2 (PC2), but also that PC1 explains an even larger fraction of the data
343 variance (**Supplementary Figure 3a**). Samples in this space have increasing Tau centroid values along
344 PC2 and increasing Oligo centroid values along PC1, indicating that these two clusters are closely
345 associated with the major axes of variation (**Supplementary Figure 3b-3g**). We further explored how
346 other clusters related to Tau, by classifying patients as negative or positive for cluster centroid values.
347 We selected a centroid cutoff value of 2x for Tau, and 1.25x or 0.8x for all other clusters that increased
348 or decreased in AD patients, respectively. Based on our initial PCA analysis, we stratified patients into
349 Tau⁻;Oligo⁻, Tau⁺;Oligo⁻, Tau⁻;Oligo⁺, and Tau⁺;Oligo⁺ groups (**Figure 2j**). We found that this classification
350 separated patients along PC1 and PC2 (**Supplementary Figure 3h**). Compared to Tau;Oligo, AD and ND
351 patients were poorly separated in PC space by either Astro;Micro or pNeuro;Neuro (**Supplementary**
352 **Figure 3i-3l**). Intriguingly, we observed that several samples were Astro⁺;Micro⁻ but no samples were
353 Astro⁻;Micro⁺ (**Supplementary Figure 3k**). This result suggests that astrocyte activation may occur before
354 microglial activation in the temporal progression of AD. Across the Tau;Oligo groups, Tau⁺;Oligo⁺
355 contained the greatest number of Astro⁺;Micro⁺ and pNeuro⁺;Neuro⁺ cases (**Supplementary Figure 3m,**
356 **3n**). Thus, Tau is most closely linked to AD diagnosis, while Tau⁺;Oligo⁺ samples have the greatest
357 amount of cellular pathologies across neuronal and glial cell types.

358 The Tau cluster may be understood in light of known AD neuropathology, but the Oligo changes
359 are less well understood and may represent an independent stage of tissue pathology. We observe that

360 the Oligo cluster includes neurofilament proteins NEFL, NEFM, and NEFH (**Figure 2d**) which are reported
361 to be markers that closely track neurodegeneration⁵⁹⁻⁶². In addition, Oligo is strongly anti-correlated
362 with the Neuro cluster which contains SYT1 and SNAP25 (**Figure 2f, 2i**), two synaptic markers of
363 neurodegeneration^{63,64}. This myelination/oligodendrocyte protein module has been found to be
364 associated with the trajectory of cognitive decline in AD²⁸ and has been observed in other forms of
365 dementia caused by cerebral atherosclerosis⁶⁵. These findings suggest that Oligo represents a general
366 remyelination response that occurs concomitant to neurodegeneration, while Tau is most closely
367 associated with development of AD-specific pathologies that precede neurodegeneration⁶⁶. In this
368 context, Tau⁺;Oligo⁻ samples are at an early stage of disease progression, while Tau⁺;Oligo⁺ samples have
369 neurodegeneration alongside Tau pathology.

370 AD pathology estimates match full proteome changes

371 To fully understand the molecular composition of clusters, we examined how peptides with
372 missing quantification values were correlated with our inferred estimates of AD pathologies taken from
373 peptide cluster centroids. We analyzed all peptides that were quantified in at least 15 samples
374 (equivalent to ≥ 2 TMT10-plex analyses) with a Spearman's rank correlation coefficient cutoff of 0.47 and
375 p-value cutoff of 5e-3. Together, these cutoffs matched fewer than 0.23% of peptides for scrambled
376 centroid values (**Supplementary Figure 4a-4d**). Using these cutoffs, a total of 2609 peptides were
377 correlated with Tau, 5554 with Oligo, 4866 with Astro, 7672 with Micro, 1863 with pNeuro, and 7200
378 with Neuro cluster centroids (**Supplementary Figure 4e-4g, Supplementary Data 2**). This analysis
379 identified a number of other phosphosites on MAPT that were correlated with Tau, including MAPT
380 pT534 (2N4R isoform: pT217), which has been identified as a specific biomarker for AD⁶⁷, as well as
381 MAPT pT529, pS531, and pS579 (2N4R isoform: pT212, pS214, and pS262) which are associated with
382 Tau's seeding activity⁶⁸ (**Supplementary Figure 4h**). For Oligo, we identified a number of other
383 oligodendrocyte-enriched proteins, such as CLDND1, CNDP1, and OPALIN that were closely associated
384 with the cluster centroid (**Supplementary Figure 4i**). Thus, we could use correlation with cluster
385 centroids to integrate our full matrix of peptide data and find further changes reflective of cluster
386 identity.

387 As there was a large degree of overlap between peptides correlated with different clusters, we
388 decided to use partial correlation to explore the relationship of peptide changes with Tau and Oligo. We
389 selected MAPT, MBP, and PLP1 as example proteins with peptides that increase in Tau⁺ and Oligo⁺
390 samples, respectively (**Supplementary Figure 5a-5d, Supplementary Data 4**). We calculated the partial
391 correlations of all peptides that were correlated with either cluster, represented in a 2D plot (**Figure 3a**).
392 We observed that among the correlated MAPT phosphopeptides, all were primarily correlated with Tau
393 (Tau partial correlation MAPT pS579: $\rho=0.91$, $p=2.7e-19$; pT534: $\rho=0.89$, $p=2.3e-17$; pS713: $\rho=0.92$,
394 $p=1.3e-16$; pS606: $\rho=0.91$, $p=2.4e-15$; **Figure 3a**). These Tau-correlated peptides were located on MAPT's
395 microtubule-binding domains and neighboring proline-rich domain (PNS Isoform: residues 468-685;
396 **Supplementary Figure 5e**). In the C-terminal domain, three MAPT phosphosites (PNS Isoform: pS713,
397 pS717, and pS721; 2N4R Isoform: pS396, pS400, pS404) were also correlated with Tau. All other
398 peptides from the N-terminal and C-terminal domains were either uncorrelated or anti-correlated with
399 Tau and Oligo, possibly due to degradation or modification with other PTMs. In contrast, peptides and
400 phosphopeptides from myelin proteins (MBP, PLP1) and neurofilaments (NEFL, NEFM, NEFH) were all
401 strongly correlated Oligo and not with Tau (Oligo partial correlation MBP: $\rho=0.98$, $p=5.3e-31$; PLP1:
402 $\rho=0.98$, $p=7.3e-28$; NEFL: $\rho=0.91$, $p=3.0e-19$; NEFM: $\rho=0.94$, $p=2.0e-22$; NEFH: $\rho=0.91$, $p=4.2e-16$; **Figure**

403 **3b, 3c, Supplementary Figure 5c, 5d).** The one exception was a peptide mapping to MBP₃₁₋₃₇ that was
404 anticorrelated with Oligo (**Supplementary Figure 5f**). This peptide is specific for the Golli MBP isoforms
405 that are only expressed during development⁶⁹. All other MBP peptides were correlated with Oligo and
406 mapped to positions after the 134 residue start site for MBP isoforms 3-6. We finally examined the
407 proteome, estimating protein abundances from median peptide levels and calculating their predicted
408 cell types. We observed that oligodendrocyte proteins were primarily correlated with Oligo, neuronal
409 proteins were primarily anticorrelated with Oligo, and microglia and astrocyte proteins were partially
410 correlated with both Tau and Oligo to varying degrees (**Figure 3d**). The anticorrelated neuronal proteins
411 included synaptic markers of neurodegeneration^{63,64} (Oligo partial correlation SYT1: $\rho=-0.86$, $p=4.7e-15$;
412 SNAP25: $\rho=-0.62$, $p=2.9e-6$). These results match the patterns of cell-type enrichment and correlation
413 between cluster centroids that we had previously observed (**Figure 2g, 2h**). Thus, our expanded
414 correlation preserves the cell-type association established with a smaller data set quantified across all
415 patients.

416 We applied a similar methodology to see if we could identify other microglial and astrocytic
417 activation markers that were correlated with their respective clusters. We find that the astrocyte
418 activation markers GFAP and VIM are strongly correlated with Astro at the level of phospho and non-
419 phospho peptides (Astro partial correlation GFAP: $\rho=0.97$, $p=1.4e-28$; VIM: $\rho=0.77$, $p=1.6e-10$; **Figure 2e,**
420 **Supplementary Figure 5g, 5h**). In contrast, the microglia-enriched protein GNA13 and activation
421 markers AIF1 (IBA1), ITGB2 (CD11B), and SPP1 were all more strongly correlated with Micro than Astro
422 (Micro partial correlation GNA13: $\rho=0.83$, $p=2.7e-13$; SPP1 pS191, pS195: $\rho=0.83$, $p=7.4e-6$; ITGB2:
423 $\rho=0.67$, $p=9.0e-5$; AIF1: $\rho=0.58$, $p=7.9e-3$; **Figure 2f, Supplementary Figure 5i-5l**). These results are
424 notable, as previous large-scale proteomics analyses have not been able to decouple astrocyte from
425 microglial changes²⁶.

426 AD pathologies are correlated with dysregulated kinase networks

427 We next examined how phosphorylation sites on different kinase, phosphatase, and receptor
428 signaling factors were correlated with our cluster centroids. Out of all phosphosites on these signaling
429 factors that were correlated with Tau, Oligo, Astro, or Micro, 85% were correlated with Tau or Oligo
430 (**Figure 4a**). To understand how known AD signaling factors were correlated with these clusters, we
431 selected a subset of known MAPT kinases, including BRSK1, BRSK2, TNIK, and TTBK1, and plotted their
432 correlation with Tau and Oligo centroids (**Figure 4b**). We observed several phosphosites on TNIK, BRSK1,
433 and BRSK2 that were primarily correlated with Tau, matching their upregulation in Tau⁺ patient groups
434 (TNIK pS680: FC=1.92, $p=2.44e-4$; BRSK1 pS443: FC=1.60, $p=4.40e-6$; BRSK2 pS423: FC=1.97, $p=6.12e-7$;
435 **Supplementary Figure 6a-6c, Supplementary Data 4**). In contrast, TTBK1 phosphosites were located
436 closer to the diagonal of the plot, reflecting that it was phosphorylated to a larger degree in Tau⁺;Oligo⁺
437 samples (TTBK1 pS483: FC=2.52, $p=5.43e-6$; **Supplementary Figure 6d**). The presence of phosphosites
438 on these kinases that are highly correlated with pathology is striking as we did not observe a
439 corresponding change in any of the non-phospho peptides, suggesting an increase in the stoichiometric
440 ratio of phosphorylation on those sites (**Supplementary Figure 6e-6h**). We applied a similar analysis to
441 the several cyclin-dependent kinases (CDKs) and observed that phosphosites on CDK5, CDKL5, CDK16,
442 CDK17, and CDK18 were correlated with both Tau and Oligo (**Figure 4c**) and significantly upregulated in
443 Tau⁺;Oligo⁺ samples (CDK5 pY15 FC=1.8, $p=1.2e-2$; CDKL5 pY171: FC=3.1, $p=2.5e-5$; CDK16 pS153:
444 FC=3.4, $p=2.9e-6$; CDK17 pS180: FC=2.9, $p=5.0e-10$; CDK18 pS132: FC=3.6, $p=2.7e-9$; **Supplementary**
445 **Figure 6i-6m, Supplementary Data 4**). For CDK5 and CDKL5, we observed that non-phosphopeptides

446 were significantly downregulated in this group, while only CDK18 had peptides that were also
447 upregulated (CDK5 FC=0.86, p=5.0e-5; CDKL5: FC=0.67, p=2.7e-3; CDK18: FC=1.5, p=9.0e-3;
448 **Supplementary Figure 6n-6r**). Among these phosphosites, CDK5 pY15 potentiates kinase activation
449 through A β ₄₂-c-Abl signaling^{70,71}; CDKL5 pY171 is an autophosphorylation site and its mutation abolishes
450 enzymatic function⁷²; CDK16 pS153 is a Protein Kinase A (PKA) substrate that blocks the protein's
451 interaction with CCNY and inhibits its function⁷³; CDK17 pS180 is elevated in A β -expressing cell lines⁷⁴.
452 While CDK5 and CDK18 are known to have altered signaling in the AD brain^{75,76}, this observation has not
453 been previously made of CDKL5, CDK16, and CDK17.

454 To aid visualization, we built a map of all kinases with phosphosites that were significantly
455 correlated with Tau or Oligo variables (**Figure 4d**). We categorized nodes based on their relative
456 correlation with Tau and Oligo. This approach highlighted proteins from all kinase families within the
457 human kinome that were associated with Tau and Oligo. Out of the 63% of these kinases that had
458 protein quantification, only 23% were significantly upregulated at a protein level in Tau⁺ or Oligo⁺
459 samples, while 46% were downregulated in these groups (**Supplementary Figure 7a, Supplementary**
460 **Data 4**). The associated kinases included MAPK8/10 (pY185/pY223: FC=2.4, p=1.3e-4), MAPK11 (pY182:
461 FC=4.7, p=1.8e-5), MAPK14 (pY182: FC=2.5, p=1.5e-5), Protein Kinase A C-subunit α/β (PKACA/B pS140:
462 FC=1.8, p=2.7e-5), Protein Kinase C α (PKCA pT228: FC=1.5, p=1.9e-3), and CAMKII $\alpha/\beta/\delta/\gamma$ (pS25/pS26:
463 FC=3.3, p=2.9e-6; **Supplementary Figure 7b-7h**), which have previously found to be activated in AD^{7,8,77-}
464 ⁷⁹. Among the phosphosites that we identified, MAPK8 pY185 / MAPK10 pY223 increase kinase activity
465 and mediate a stress response⁸⁰. Similarly, MAPK14 pY182 is necessary for kinase activity and mediates a
466 stress response⁸¹ and MAPK11 pY182 has a high degree of homology to MAPK14 that likely confers
467 similar functional characteristics. PKACA/B are subunits of another kinase that phosphorylates Tau⁷, and
468 pS54 and pS140 are both autophosphorylation sites on these proteins⁸². PKCA is a kinase that mediates
469 synaptic pathologies caused by A β ⁸; PKCA pT228 is phosphorylated by MST2 and is necessary for its
470 activation in mice⁸³. For CAMKII, we observe several phosphosites on its protein subunits that are
471 significantly upregulated in Tau⁺;Oligo⁺ samples, including pY17, pS25, pS71, pS145, pS257, and pS276,
472 even as total protein levels of CAMKII subunits are significantly downregulate in this same group
473 (**Supplementary Figure 7h, Supplementary Data 4**). While most of these phosphosites are of unknown
474 function, pT306 has been reported to block CAMKII's interaction with Calmodulin, inhibiting its kinase
475 activity⁸⁴. Additionally, CAMKII's pT286 autophosphorylation site⁸⁵ is downregulated in this group and
476 correlated with pNeuro, indicating that CAMKII has reduced total activity in samples with neuronal
477 pathology (**Supplementary Figure 7h, Supplementary Data 2**). These findings point to a complex role of
478 phosphorylation PTMs in regulating CAMKII activity in AD.

479 We next examined whether other kinase associations represented novel insight. Within the
480 tyrosine kinase family tree, SRC-family kinases (SFKs) act as key signaling nodes in mediating tyrosine
481 receptor signaling⁸⁶. We observed increased phosphorylation on SRC in Tau⁺;Oligo⁺ samples (pS75:
482 FC=1.5, p=1.2e-3; pY187: FC=1.4, p=6.0e-5; pY439*: FC=2.7, p=1.2e-3; **Supplementary Figure 7i**). SRC
483 pS75 has been identified as a substrate of CDK5 that inhibits SRC function⁸⁷. While the exact function of
484 SRC pY187 is not currently known, the site is on SRC's SH2 domain and bears strong sequence homology
485 to similar autoinhibitory sites on other SFKs⁸⁸. Alongside SRC pS75, we see that LMTK2, another CDK5
486 substrate⁸⁹, has increased phosphorylation in Tau⁺;Oligo⁺ samples (pS1450: FC=1.6, p=6.4e-3;
487 **Supplementary Figure 7j**). Downstream of SRC, we observe that PTK2 has increased phosphorylation in
488 this same group (pY925: FC=1.6, p=2.9e-4; **Supplementary Figure 7k**). This phosphosite is a SRC-specific

489 substrate that is necessary for PTK2's regulation of cell motility in human cell lines⁹⁰. Among the other
490 tyrosine kinases, we observed that DDR1 has increased phosphorylation in both Tau⁺ and Oligo⁺ samples
491 (pY796: FC=2.1, p=3.1e-4; pY792: FC=1.6, p=5.5e-3; **Supplementary Figure 7a, 7l**). These two
492 phosphosites are on the conserved activation loop of DDR1⁹¹ and inhibition of this receptor has recently
493 been shown to decrease inflammation in mouse models of AD and Parkinson's disease⁹²⁻⁹⁴. We also
494 observed that an EGFR autophosphorylation site³³ is increased in Tau⁺;Oligo⁺ samples (pY1175: FC=2.9,
495 p=9.4e-4; **Supplementary Figure 7m**). In total, we find that our collection of Tau- and Oligo-correlated
496 phosphosites includes a number signaling factors that are known to be active in AD as well as novel
497 associations on sites linked to increased kinase activity.

498 **AD kinase networks overlap with substrate and mouse model phosphoproteomes**

499 To test whether the phosphosites downstream of the pathology-associated kinases were also
500 dysregulated, we applied phosphosite enrichment analysis (PSEA) using publicly available kinase-
501 substrate datasets⁵⁶. By calculating enrichment scores for different cluster correlates, we find that
502 phosphosites regulated by BRSK1, BRSK2, CDK5, PKACA, CDK1, CAMK2A, PKCA, GSK3B, ERK1, and ERK2
503 are associated with one or more cluster centroids (**Figure 4e**). These findings are supported by the direct
504 association of phosphosites on BRSK1 and BRSK2 (**Supplementary Figure 6f, 6g**), CDK5 (**Supplementary**
505 **Figure 6n**), CAMK2A (**Supplementary Figure 7a**), PRKCA (**Supplementary Figure 7a**), GSK3A/B (**Figure 2f,**
506 **Supplementary Data 2**), and MAPK3 and MAPK1 (ERK1 and ERK2; **Supplementary Data 2**) with the most
507 strongly enriched clusters. In the case of PKACA, we find that some downstream phosphosites increase
508 with Tau, Astro, and Oligo, while others decrease with pNeuro (**Supplementary Figure 8a-8d**). Among
509 the Tau- and pNeuro-correlated PKACA substrates, CAMKK1 pS475 and pS52 are increased and
510 suppressed, respectively, in the presence of Ca²⁺/Calmodulin⁹⁵. Many of the Astro- and Oligo- correlated
511 PKACA phosphosites are located on astrocyte- and oligodendrocyte-enriched proteins, suggesting that
512 this kinase also dysregulated these cell types as well. Altogether, we find that kinase phosphosites are
513 correlated with substrate phosphopeptides changes and reflect signaling pathways known to be
514 activated in AD. Our data strengthens support for these factors and adds a new layer with several novel
515 kinase associations.

516 We finally examined the overlap between the phosphoproteomes of AD and three mouse
517 models of neurodegeneration²². In this previously collected dataset, we analyzed the phosphoproteome
518 of CK-p25, 5XFAD, and Tau P301S mouse brain tissue using a similar methodology to this study. Out of
519 all of the differentially phosphorylated sites in mice, 96.7% could be mapped by homology to human
520 proteins (**Supplementary Data 5**). We observed that the CK-p25 mouse model with two weeks of
521 p25/Cdk5 activation had the greatest number of overlapping phosphosites that shared directional
522 changes with AD pathologies (**Supplementary Figure 8e**). Interestingly, MAPT emerged as the one
523 protein with phosphorylation sites that were associated with pathology in each mouse model and in
524 human patients (**Figure 4f-4g, Supplementary Figure 8f**). We observed a number of aforementioned
525 signaling factors that also had phosphosites enriched in one or two mouse models, including BRSK1,
526 BRSK2, CAMKII, CAMKV, and PTPRZ1. This comparison also allows us to tie phosphorylation on these
527 signaling factors to pathologies driven by p25/Cdk5, A β , or phospho-MAPT activation in each respective
528 mouse model. These results may inform the future choice of mouse models to study particular signaling
529 pathway dysregulation events.

530 Statistical modeling links the phosphoproteome to AD histopathologies

531 To better understand the associations between peptide changes and histopathology variables,
532 we generated a series of partial least squares regression (PLSR) models. We used PLSR to link our cluster
533 centroids to each component of the full matrix of histopathology measurements (**Figure 5a**,
534 **Supplementary Data 1**). We first aimed to reduce the size of the histopathology matrix to variables that
535 were relevant to the cortex tissue that we analyzed. By building separate PLSR models with K-fold cross
536 validation analysis for each outcome in the histopathology matrix, we determined that a subset of
537 pathology variables could be predicted by PLSR from peptide data with moderate accuracy (*e.g.*
538 Consortium to Establish a Registry for Alzheimer Disease neuropsychological battery (CERAD-NP, NIA-
539 Reagan (NIA-R), plaque score, and temporal tangles (TangleT); $Q^2 \cong 0.39 - 0.60$) (**Figure 5b**). Although
540 our models do not have predictive accuracy for AD risk variables such as APOE genotype or White
541 Matter scores, our patient cohort did not have any cases of the most severe APOE $\epsilon 4/\epsilon 4$ alleles and
542 there are few cases with White Matter score > 0 in the temporal lobe where we sampled tissues.

543 In order to interpret the covarying relationship between peptide cluster centroids and pathology
544 outcomes, we generated a PLSR model linking cluster centroids to a multivariate Y block matrix
545 composed of the non-redundant predictable pathology variables (CERAD-NP, NIA-R, plaque score, and
546 TangleT). We found that a 2-component PLSR model explained $\sim 57\%$ of the data variance for both X and
547 Y matrices (**Figure 5c**). We then plotted this model's X scores, X weights, and Y loadings vectors in latent
548 variable (LV) space to visualize how centroid and pathology measurements covary. Samples were
549 separated by their X scores values on LV1 based on Tau status and LV2 based on Oligo status (**Figure 5c**).
550 We examined the X weight vectors for this model and found that the Tau, Astro, Oligo, and pNeuro
551 centroids all strongly contributed to the final model (VIP score > 1 ; **Figure 5d**). In LV space, Tau, Astro,
552 and pNeuro weight vectors strongly contributed to LV1 separation, while Oligo was the strongest
553 contributor to LV2 separation (**Figure 5d**). Furthermore, the Tau/Astro and pNeuro clusters have roughly
554 opposite directions from the origin (**Figure 5d**), in agreement with these groups being upregulated and
555 downregulated respectively in AD patients (**Figure 2j**, **Supplementary Figure 3k, 3l**). Finally, we
556 examined the Y loadings vectors for this model and found that plaque score, TangleT, NIA-Reagan, and
557 CERAD-NP all occupy the same LV space (**Figure 5e**, **Supplementary Figure 9a-8c**). We tested the
558 accuracy of this multivariate PLSR model and found that it had a similar predictive accuracy to the single-
559 outcome models (**Supplementary Figure 9d**). Overall, our regression analysis allows us to relate our
560 cluster centroids to patient AD histopathology measurements. Intriguingly, there were not any
561 histopathology variables that were strongly associated with the negative direction of Oligo changes on
562 LV2, yet AD patients were well distributed along the LV2 axis between Tau⁺ (high LV2) and Oligo⁺ (low
563 LV2). These results highlight the need for post-mortem characterization of oligodendrocyte pathology
564 alongside other traditional AD pathologies.

565 To understand which individual peptides were most predictive of AD pathologies, we built a
566 series of peptide-centric multivariate PLSR models. We find that peptide-centric models give similar
567 patient-histopathology association scores as the model built on cluster centroids (**Supplementary Figure**
568 **9e**). To visualize the relation between peptides and histopathologies, we rank ordered peptides by their
569 association with AD pathologies in LV space (**Supplementary Data 6**). We first analyzed a model
570 generated from pTyr peptides measured in all samples and found that creatine kinase B-type (CKB) pY39
571 was the most strongly associated pTyr peptide with pathology (**Figure 5f**). Focal creatine deposits have
572 been observed in AD brains⁹⁶, and CKB is reported to have post-translation oxidation and downregulated

573 activity in AD⁹⁷. Creatine and cyclocreatine have been shown to have some positive effects in mouse
574 models of neurodegeneration and have been suggested as a dietary supplements to offset the loss of
575 CKB activity⁹⁸⁻¹⁰⁰. Alongside CKB pY39, we found several other phosphosites on CKB that are increased in
576 Tau⁺ samples even as total CKB protein does not change (**Supplementary Figure 9f**). These phosphosites
577 may contribute to CKB's reduced activity, although their exact function is not yet known. IQSEC2, a
578 component of the NMDA receptor complex which is overactivated by A β in AD^{101,102}, was also associated
579 with pathology. Phosphosites on CDKL5 and DDR1 were also associated with AD (**Figure 5f**),
580 strengthening our findings from kinase correlation analysis. In the other direction, we saw that
581 phosphosites on the synaptic proteins ANK2 and SHANK3 as well as phosphosites on the activation loops
582 of MAPK1 and MAPK3 were negatively associated with AD (**Figure 5f**). This finding matches the
583 prediction from PSEA that ERK1 and ERK2 substrates were associated with pNeuro and Neuro (**Figure**
584 **3i**). This contrasts with MAPK1 and MAPK3 being significantly upregulated at the level of total protein in
585 Tau⁺;Oligo⁺ samples (**Supplementary Data 4**), suggesting that ERKs are less activate at a bulk tissue level
586 in AD. The upregulation of ERK proteins may be a compensatory mechanism or due to differences in ERK
587 regulation in different cell types in the brain. While acute A β stimulation has been shown to activate the
588 ERKs in neurons, the total level of phosphorylation on the activation loops of ERK is highly variable in AD
589 tissues¹⁰³ and had not be previously associated with specific disease processes.

590 In a second PLSR model built from pSer/pThr peptides, we found that phosphosites on MAPT
591 were among those most strongly associated with AD pathologies (**Figure 5g**). These sites include the
592 aforementioned MAPT pS579, pS713, and pT534 phosphosites that were strongly correlated with the
593 Tau centroid. We also observed that Paralemmin-1 (PALM) pT141 and pT145 are strongly associated
594 with AD (**Figure 5g**). PALM is a modulator of plasma membrane dynamics¹⁰⁴ with an uncharacterized role
595 in AD. In the other direction, the pNeuro cluster peptides CAMKK1 pS52 and CASKIN1 pS935 were
596 among the most negatively-associated phosphopeptides in this model (**Figure 5g**). Compared to the
597 conventional synaptic markers SYN1 and SYT1 which are significantly downregulated only in Neuro⁺
598 samples, these phosphosites were primarily downregulated in pNeuro⁺ samples (**Supplementary Figure**
599 **9g-9j**). These findings indicate that the neuronal synaptic phosphoproteome is affected in AD, even in
600 the absence of total synaptic protein downregulation.

601 In a final PLSR model built on the proteome, we find that the secreted glycoprotein EMILIN3 and
602 complement protein C4A were the proteins most strongly associated with pathology (**Figure 5h**). Of
603 these two proteins, EMILIN3 was specifically upregulated only in Tau⁺ samples (**Supplementary Figure**
604 **9k-9l**). Little is known about EMILIN3 in the context of the brain, and, to our knowledge, this is the first
605 report of its association with AD. VGF, a marker reported to be downregulated in AD^{28,105}, was the most
606 negatively-associated protein with pathology in this model and was significantly downregulated only in
607 Tau⁺ samples (**Supplementary Figure 9m**). ROC analysis of the proteins and phosphosites highlighted by
608 our PLSR models finds they are each predictive of AD status (**Supplementary Figure 9n**). Overall, our
609 analysis recapitulates known protein and phosphopeptide links to AD and reveals new markers and
610 signaling factors, such as the kinases CKB, CDKL5, and DDR1, that are closely associated with disease.

611 Discussion

612 In this study, we used phosphoproteomics to explore cellular signaling network changes in AD.
613 We followed a data collection strategy that enabled the quantification of low-abundance pTyr
614 phosphopeptides alongside the pSer/pThr phosphoproteome and proteome from 48 AD and age-

615 matched control patients. By capturing a deep molecular profile of the phosphoproteome alongside the
616 proteome for individual patient samples, we were able to build a map of how signaling factors become
617 activated alongside other pathological processes. Our analysis uncovered co-correlated sets of peptides
618 including: (1) “Tau,” a cluster associated with Tau pathology and (2) “Oligo,” a cluster containing
619 oligodendrocyte, myelination, and neurofilament proteins. Tau was closely associated with AD
620 diagnosis, while Oligo was more closely associated other cellular pathology peptide modules. In
621 addition, we identified neuronal phosphopeptide and protein modules that were negatively associated
622 with Tau and oligodendrocyte pathologies. We observed phosphosites on several AD-associated kinases
623 that were correlated with pathological changes, as well as kinase-substrate networks that followed
624 similar trends. We additionally found several novel kinase phosphosites that were correlated with these
625 pathologies, including CDKL5, CDK16, and CDK17, as well as tyrosine kinases SRC, EGFR, DDR1, and PTK2.
626 Our dataset adds a new layer of understanding on how these signaling factors correlate with early- and
627 late-stage AD pathologies.

628 Although our analysis was of bulk tissue, many of the differentially expressed proteins were
629 found to be primarily expressed in glial cells. Among the previously reported markers of
630 neurodegeneration^{60–64,106}, we observed that the Oligo cluster is strongly correlated (Spearman $\rho > 0.9$)
631 with neurofilament proteins and strongly anticorrelated (Spearman $\rho < -0.7$) with synaptic proteins. One
632 major question arising from our work is the nature of this myelination response. Previous proteomics
633 analyses have linked myelination to cerebral atherosclerosis as well as the trajectory of cognitive decline
634 in AD^{26,28,65}. In addition, a subset of these proteins were found to be upregulated at a transcript level in
635 single-nuclei RNA-seq analyses of AD oligodendrocytes⁵⁴. A previous analysis of a mouse model of
636 tauopathy has found that oligodendrogenesis increases in hippocampal grey and white matter regions
637 prior to locomotive and memory impairments¹⁰⁷. We have similarly observed increased phosphorylation
638 on PLP1 by 6 months of age in the Tau P301S model. However, our AD proteomics analysis also finds a
639 number of Oligo⁺ patients without Tau pathology. This suggests that myelination occurs as a general
640 response concomitant to neurodegeneration downstream of Tau pathology alongside other neurotoxic
641 insults.

642 Overall, our analysis highlights the importance of integrating the phosphoproteome with
643 proteome and transcriptome datasets. While our proteome dataset captured changes in
644 oligodendrocyte and neuronal protein abundances, the pSer/pThr and pTyr phosphoproteomes were
645 necessary to directly measure Tau pathology as well as a downregulated neuronal phosphopeptide
646 module. The phosphoproteome also provides direct measurement of the regulatory sites on protein
647 kinases and other signaling factors. We identified a number of proteins that are associated with AD at
648 the level of phosphorylation even as the proteins themselves are unchanged or downregulated. These
649 phosphorylation changes were most likely to be associated with Tau pathology, supporting the
650 hypothesis that protein phosphorylation events mediate signaling events that are pre-emptive of
651 neurodegeneration. Our dataset allows us to separate signal events associated with early- vs. late-stage
652 AD pathologies as well as other forms of neurodegeneration. By targeting the signaling events occurring
653 at an early-stage of AD, we may be able to delay or prevent disease progression.

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667 Author Contributions

668 N.M. and F.W. designed the study. D.M. collected human brain tissue samples. N.M. and F.H.R. collected
669 proteomics data sets. N.M. wrote proteomics data integration and enrichment software. N.M. and M.L.
670 performed statistical analyses. N.M., M.L., and F.M.W. wrote the manuscript.

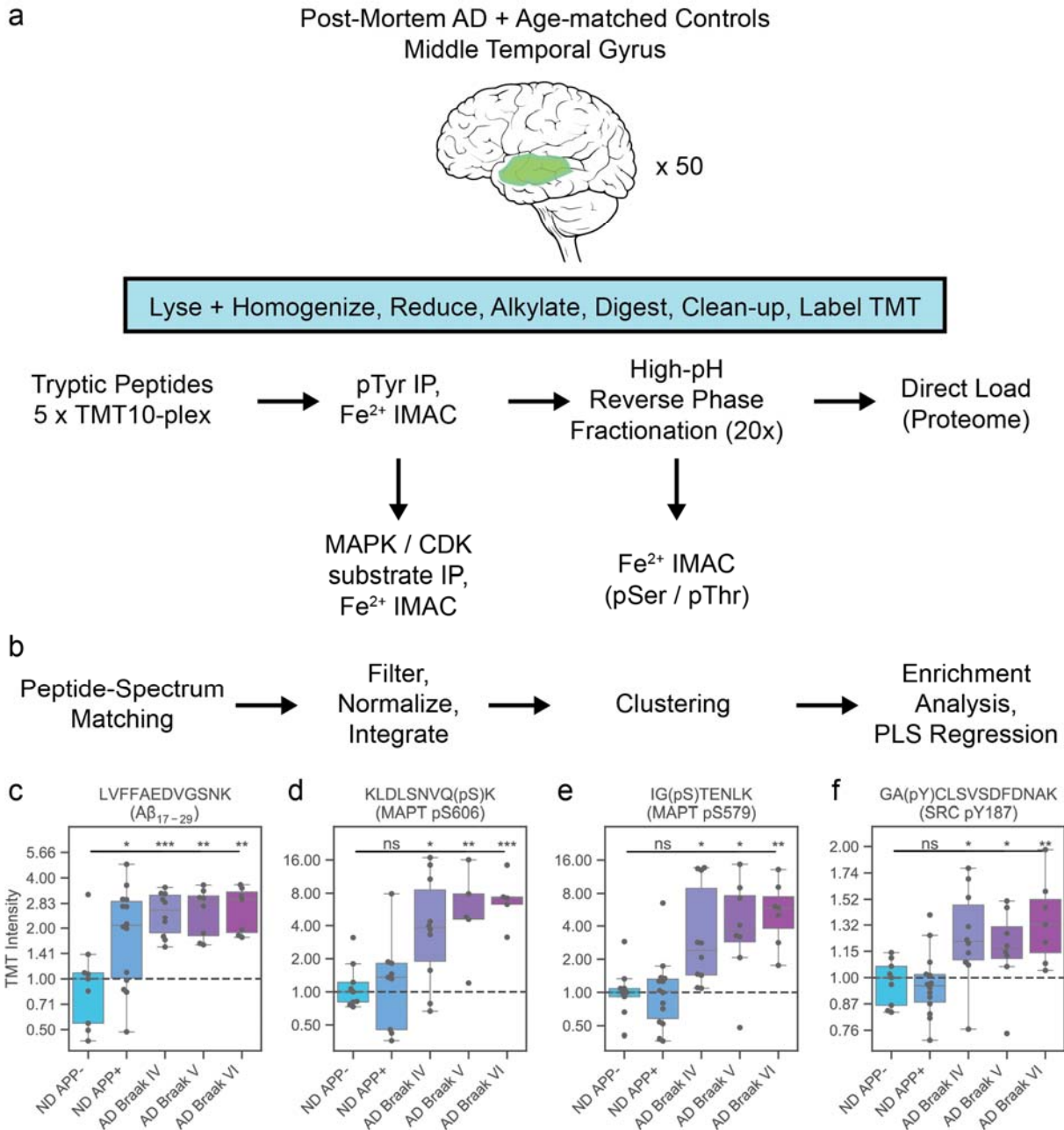
671 Competing interests

672 The authors declare no competing interests.

673 Materials & Correspondence

674 Correspondence and requests for materials should be addressed to F.M.W. (email: fwhite@mit.edu).

675 **Figures**



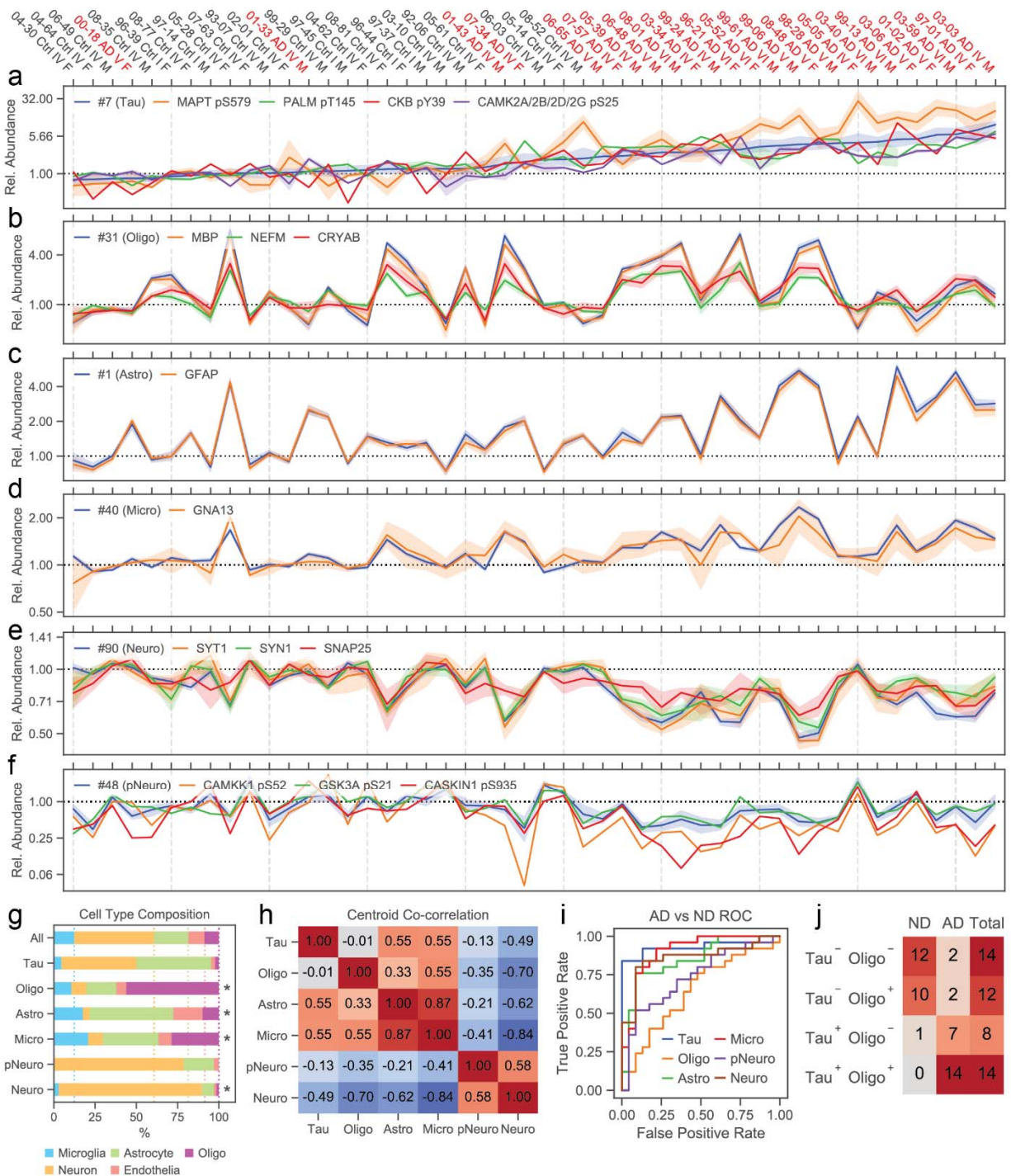
677 **Figure 1. Combined phosphoproteomics and proteomics analysis captures molecular signaling profile**
678 **of Alzheimer's disease.**

679 (a) Workflow describing proteomics sample processing and peptide enrichment. Frozen patient tissue
680 was lysed and homogenized to extract proteins and then reduced, alkylated, and digested into short
681 tryptic peptides. Proteolytic digests were then labeled with TMT10-plex for multiplexed quantification.
682 pTyr peptides were pulled down by antibody and cleaned up using IMAC. IP supernatants were then IP'd
683 for MAPK-CDK substrates and then fractionated offline at high-pH into 20 concatenated fractions.
684 Fractions were then direct-loaded onto an autosampler for global proteomics and then enriched for

685 global pSer/pThr peptides using IMAC. All enriched peptides were then analyzed on a Q Exactive mass
686 spectrometer to identify and quantify the relative abundance across samples.

687 (b) Raw mass spectrometry data was searched using ProteomeDiscoverer and MASCOT to generate
688 peptide-spectrum matches (PSMs). PSMs were filtered to remove low-quality matches and TMT
689 abundances were normalized to compare across 10-plex runs. PSMs matching the same peptide
690 sequence were then merged together to generate a final peptide abundance matrix. Peptide data were
691 analyzed via K-means clustering and combined with clinical pathology assessments using PLSR modeling
692 to identify covarying data trends. Final peptide modules were used for gene-set, kinase-substrate set,
693 phosphorylation-motif, cell-type and PPI-enrichment analyses to identify network-level trends.

694 (c-f) Selected trends are shown for peptides matched to (c) A β_{17-28} , (d) MAPT pS606, (e) MAPT pS579,
695 and (f) SRC pTyr187. Each plot shows peptide fold changes for integrated TMT values. Values are
696 normalized to the median of ND APP- patients. Stars indicate t-test values for each group with ND APP-
697 patients. ns, not significant ($p > 0.05$); ** $p < 1e-2$; *** $p < 1e-3$; **** $p < 1e-4$.



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Figure 2. Clustering analysis identifies pathology-associated peptide modules.

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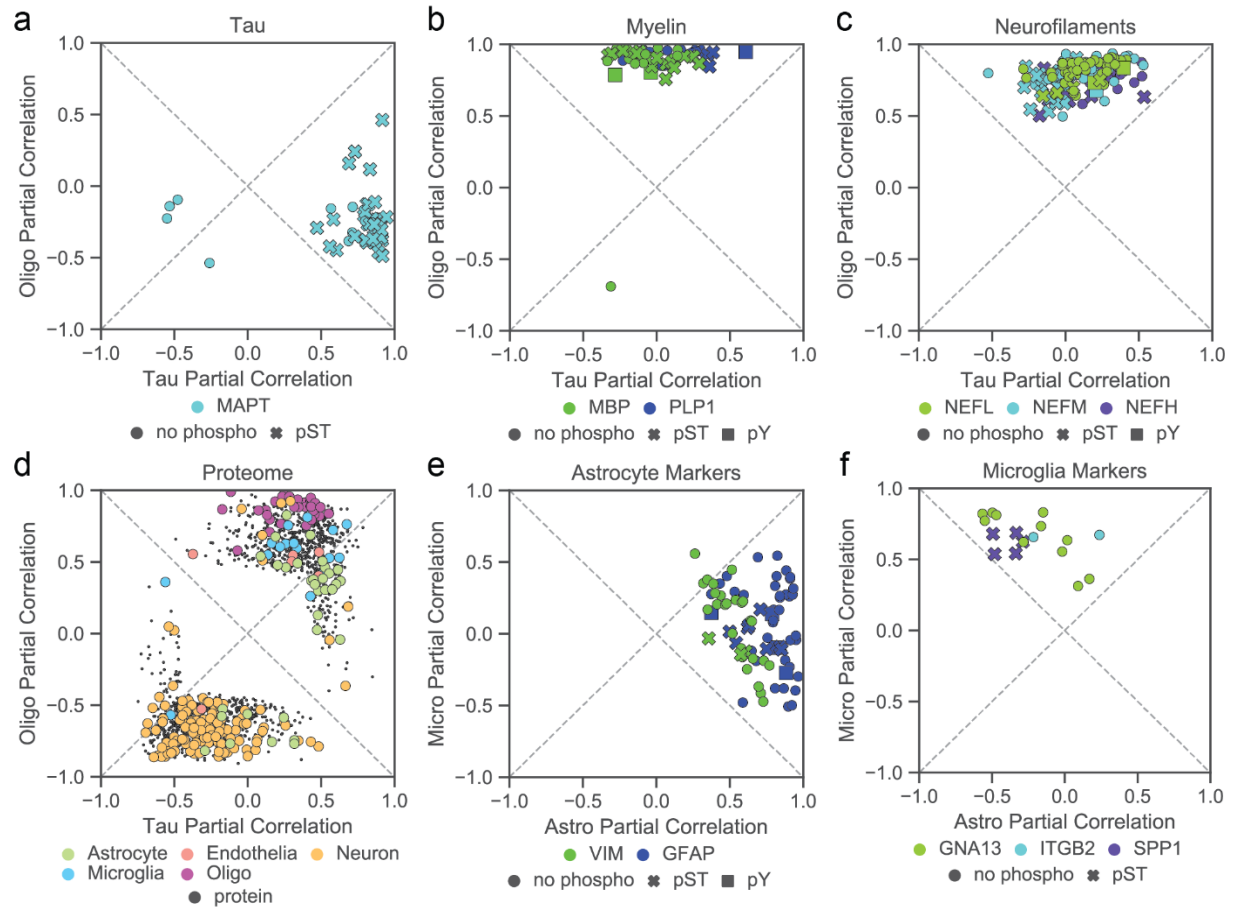
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(a-f) Relative TMT levels for Mini-Batch K-Means cluster member peptides and selected phosphosites and proteins most correlated with each cluster centroid. Line plots show cluster centroids and member peptides for (a) Tau, (b) Oligo, (c) Astro, (d) Micro, (e) Neuro, and (f) pNeuro clusters. Line plots indicate 95% confidence intervals for all cluster peptides, all redundant phosphosites, or all peptides without PTMs (proteins). Samples for all plots are ordered by Tau centroid value shown in (a).

- 705 (g) Predicted cell type composition of proteins associated with cluster centroids. Peptides seen in ≥ 15
706 patients are used for calculation. Proteins were considered if they had at least one peptide associated
707 with cluster centroids. * indicates that cluster-associated proteins had significantly different cell type
708 compositional make-up (χ^2 contingency $< 1e-3$) compared to the entire dataset.
- 709 (h) Spearman's correlation coefficients between selected cluster centroids.
- 710 (i) ROC curve predicting AD status from each named cluster centroid.
- 711 (j) Patients numbers for Tau⁺ and Oligo⁺ patient stratification generated from Tau and Oligo cluster
712 centroid values.



713

714 **Figure 3. Correlation analysis finds pathology-associated change in the full peptidome.**

715 (a-c) Partial correlation with Tau and Oligo cluster centroids for (a) MAPT peptides, (b) myelin proteins,
716 and (c) neurofilament proteins. Only peptides that are seen in ≥ 15 patients and significantly correlated
717 with either Tau or Oligo are shown. Points are colored by protein ID and stylized by the presence of
718 PTMs. Dotted lines on the diagonal are shown as reference for relative correlation.

719 (d) Partial correlation with Tau and Oligo for all protein levels estimated from all non-phospho peptides.
720 Points are colored by proteins' predicted cell type.

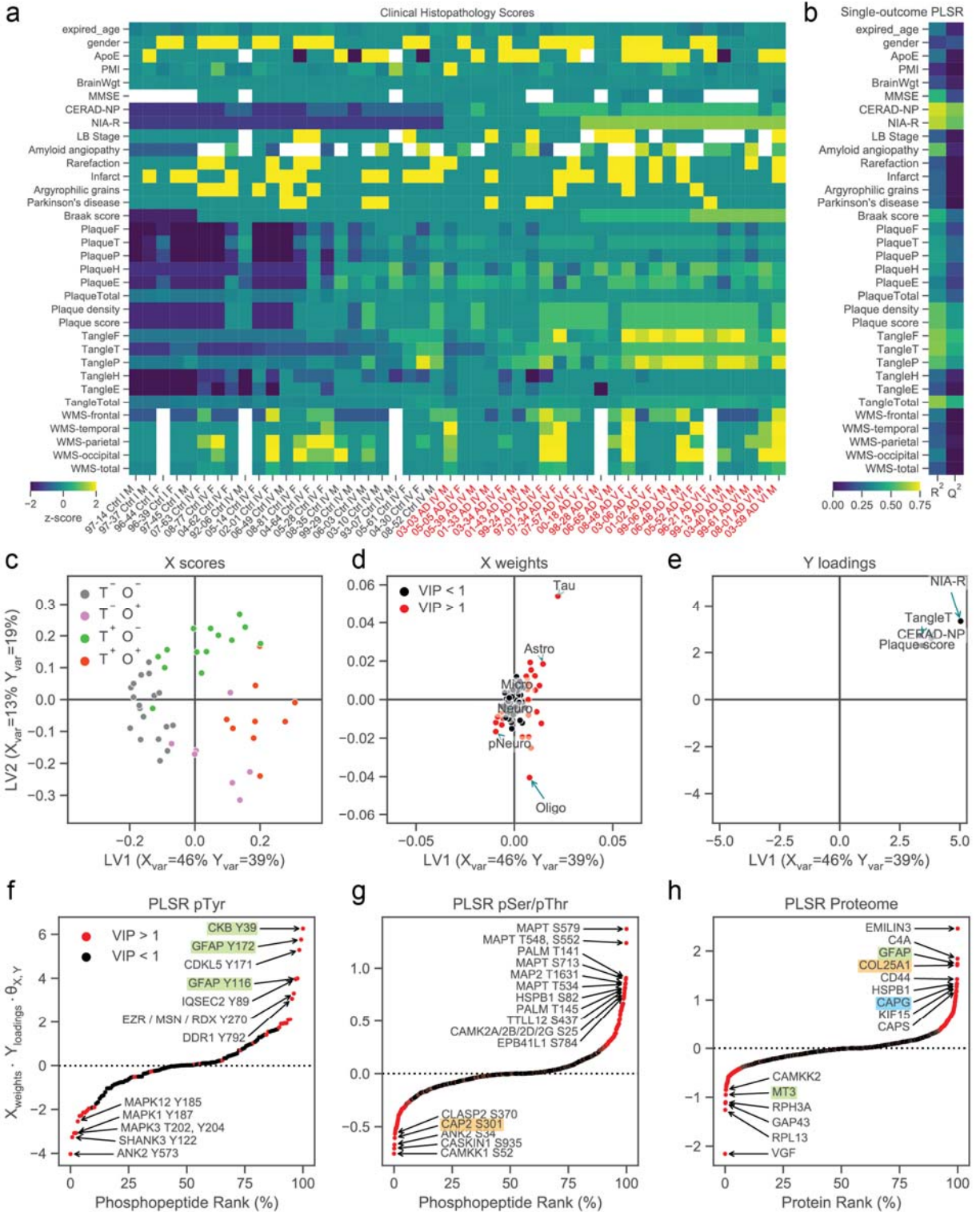
721 (e-f) Partial correlation with Astro and Micro for selected (e) astrocyte-enriched and (f) microglia-
722 enriched peptides.

727 (b-c) Partial correlation with Tau and Oligo for selected (b) Tau kinase and (c) cyclin-dependent kinases.

728 (d) Kinome map of all phosphopeptides that were significantly associated with Tau or Oligo cluster
729 centroids. Branches and nodes are colored by partial correlation with Tau and Oligo as shown on inset
730 panel.

731 (e) Phosphosite enrichment analysis applied to the phosphoproteome dataset for each cluster centroid.
732 Normalized-enrichment scores are shown for phosphosite sets that were significantly associated with at
733 least one cluster centroid. Circle sizes correspond to the number hits for each phosphosite set.

734 (f-g) Phosphoproteins that were identified to have at least one shared phosphosite with three mouse
735 models of neurodegeneration. Proteins are shown as a Venn diagram between upregulated
736 phosphosites and (f) Tau or Astro and (d) Micro or Oligo cluster centroids. Numbers indicate the total
737 number of phosphoproteins in each overlapping set and selected protein names are shown.



738

739 **Figure 5. PLSR model ties clusters and peptides to AD histopathologies.**

740 (a) Clinical histopathology measurements analyzed by PLSR for each patient. Rows are z-score

741 normalized and missing values are shown as white.

742 (b) K-fold validation of single-outcome PLSR models for each histopathology variable. R^2 and Q^2 values
743 are shown for training and test data predictions respectively.

744 (c-e) Multivariate PLSR model using cluster centroids and select clinical histopathology variables. (c) X
745 scores, labeled by Tau (T) and Oligo (O) status, (d) X weights, and (e) Y loadings vectors are shown in
746 latent variable (LV) space. The percent variance explained by the first two components of the model for
747 the X and Y matrices are shown on the axis labels.

748 (f-h) Peptide-pathology association scores generated from peptide-centric multivariate PLSR models
749 built from (f) pTyr peptides, (g) pSer/pThr peptides, and (h) estimated protein abundances.

750 **Supplementary Information**

751 **Supplementary Data 1. Demographic information and clinical histopathology measurements for**
752 **individual patients.**

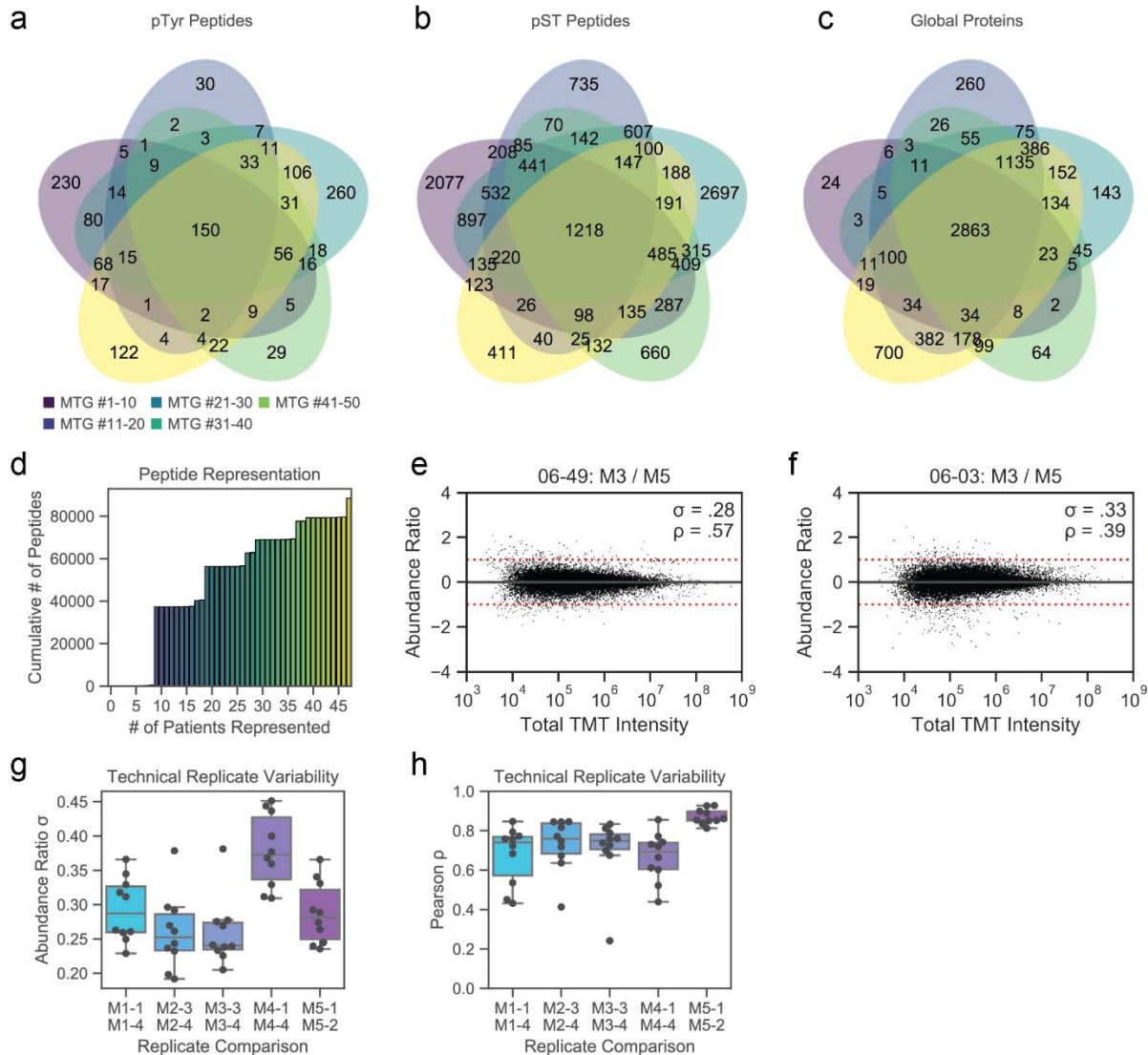
753 **Supplementary Data 2. Proteome and phosphoproteome cluster correlations.** Table includes the
754 spearman's correlation and associated p-values between each peptide and each cluster centroid.

755 **Supplementary Data 3. Peptides cluster identities.** Cluster identities and centroid values for peptides
756 quantified in all samples.

757 **Supplementary Data 4. Proteome and phosphoproteome cluster fold changes.** Table includes fold
758 changes and p-values for each peptide and cluster group against their respective negative cases.

759 **Supplementary Data 5. Mouse phosphoproteome overlap.** AD mouse model phosphosites from
760 Morshed et al. (2020) that had homologous phosphosites detected in the human AD phosphoproteome.

761 **Supplementary Data 6. Peptide scores and VIP values for peptide-centric PLSR models.** Table includes
762 tabs for PLSR models built from pTyr, pSer/pThr, Protein, and all data.



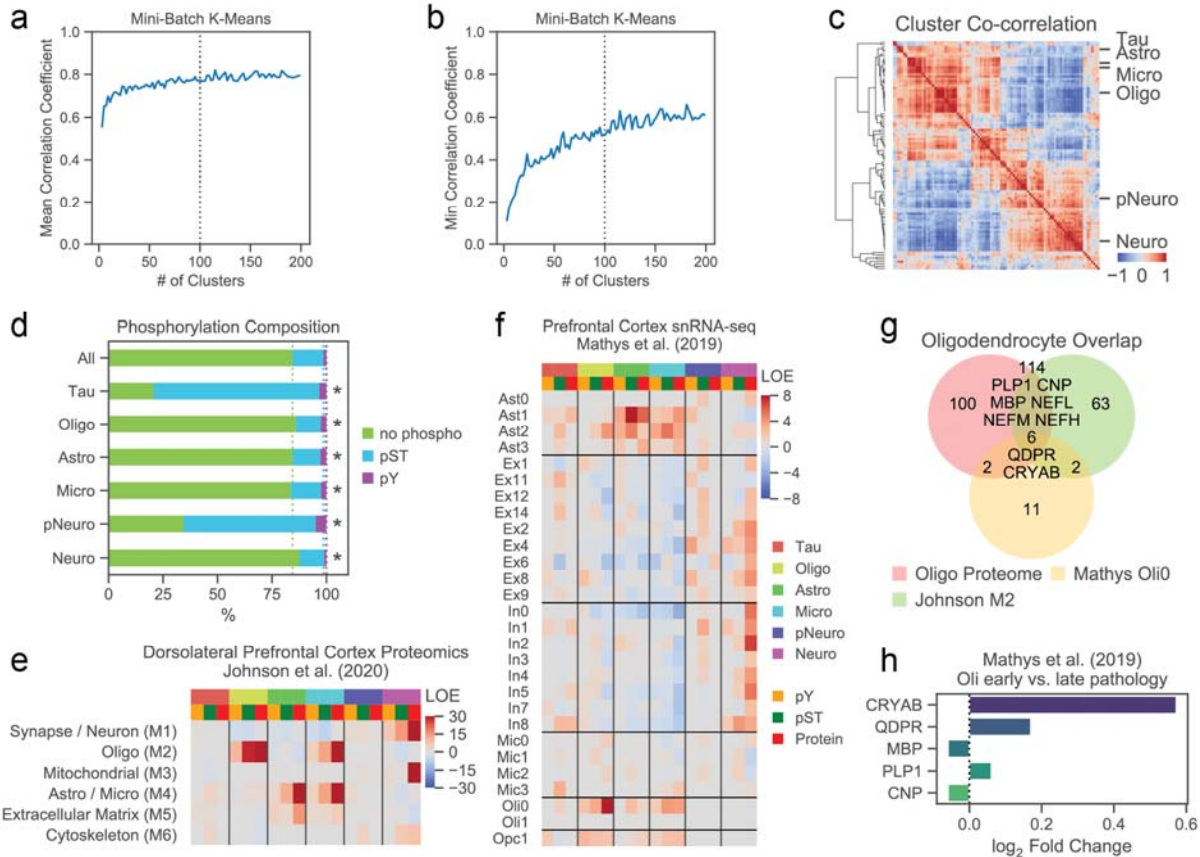
763
 764 **Supplementary Figure 1. Proteome and phosphoproteome dataset statistics and quantification**
 765 **reproducibility.**

766 (a-c) Venn diagram showing peptide overlap between (a) pTyr sites, (b) pSer/pThr sites, and (c) proteins
 767 identified in each 10-plex analysis.

768 (d) Cumulative number of peptides identified compared to the number of patients with peptide
 769 quantification.

770 (e-f) Reproducibility analysis of peptide quantification after data integration and normalization. Show
 771 are the \log_2 quantification ratios between two separate brain samples from patients (e) 06-49 and (f) 06-
 772 03. Peptides are separated on the x-axis by \log_{10} total TMT intensity from all PSMs in both 10-plex
 773 analyses. The standard deviation of abundance ratios and Pearson's correlation coefficient between all
 774 peptide replicates are listed in the panels.

775 (g-h) Reproducibility of peptide quantification between technical replicates for each TMT10-plex
776 analysis. Shown are (g) the standard deviation of abundance ratios and (h) the Pearson's correlation
777 coefficient calculated over all peptides.



778
779 **Supplementary Figure 2. Accessory clustering and dataset overlap analyses.**

780 (a-b) Properties of clusters generated by clustering with increasing number of clusters. Shown are the (a)
781 mean Pearson correlation coefficient for cluster peptides with their respective cluster centroid,
782 averaged over all clusters, and (b) minimum absolute Pearson correlation coefficient for cluster peptides
783 with their respective cluster centroid, summed and normalized over all clusters. Dotted line indicates
784 the final cluster count used for downstream analysis.

785 (c) Co-correlation heatmap between all cluster centroids, group by cluster. Selected named clusters are
786 shown on the y-axis.

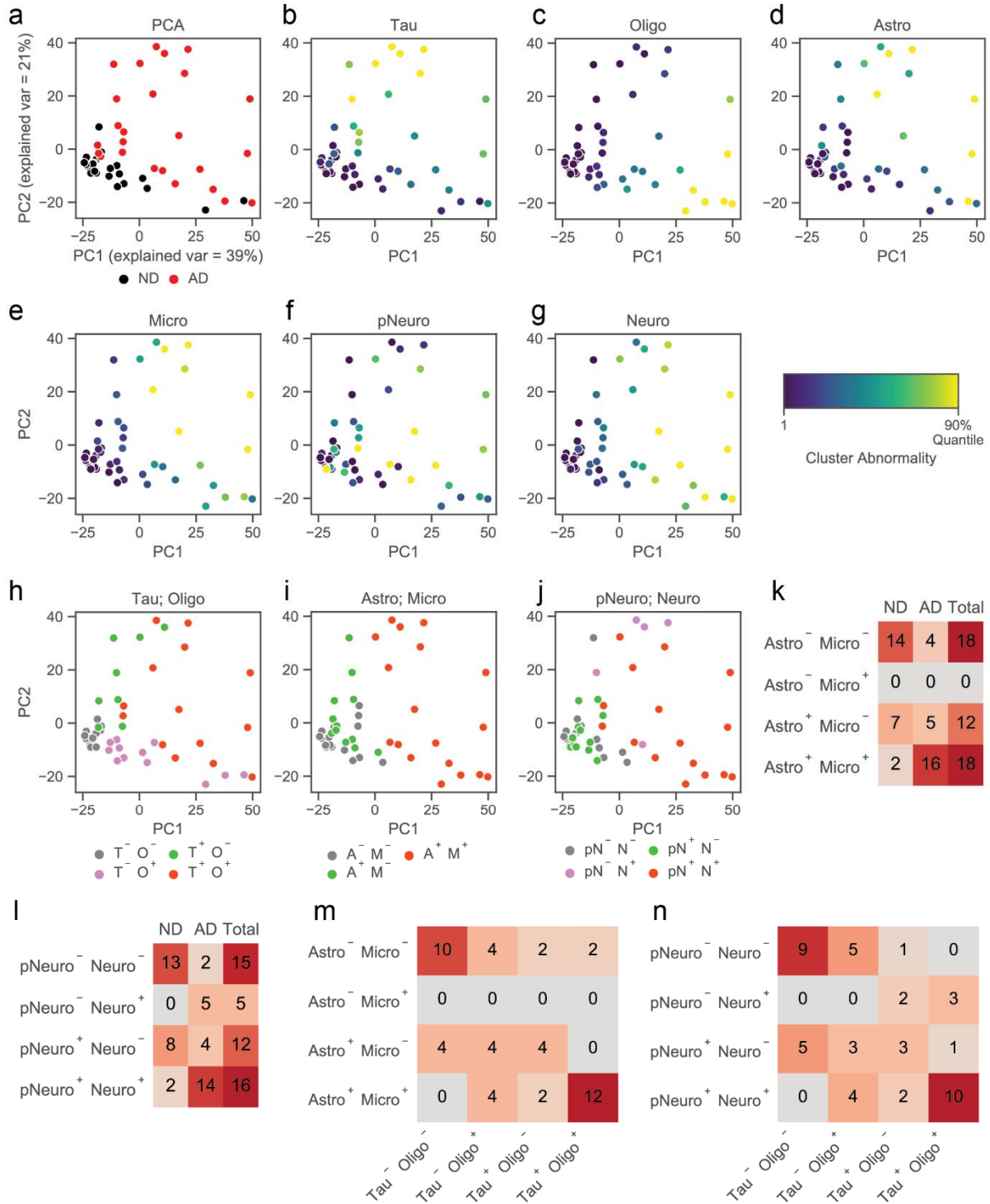
787 (d) Phosphopeptide composition of all peptides associated with cluster centroids. Peptides are labeled
788 as ‘pY’ if they contain at least one pTyr site, ‘pST’ if they contain at least one pSer or pThr site, or
789 ‘no phospho’ if they are not phosphorylated. Statistics calculations are same as **Figure 2g**.

790 (e) Binomial enrichment for proteome module marker proteins identified in Johnson et al. (2020).
791 Heatmap shows log-odds enrichment (LOE) values for the overlap of marker transcripts with pTyr,
792 pSer/pThr, and proteome datasets that are correlated with cluster centroids.

793 (f) Binomial enrichment for single-nuclei populations markers identified in Mathys et al. (2019). Legend
794 is same as (e).

795 (g) Venn diagram overlap between proteins correlated with Oligo in our dataset, M2 Oligo proteome
796 module from (e) and Oli0 marker transcripts from (f). Numbers indicate the total number of proteins in
797 each overlapping set and selected protein names are shown.

798 (h) Transcript \log_2 Fold Change values Oli0 population from Mathys et al. (2019) for markers of the Oligo
799 cluster.



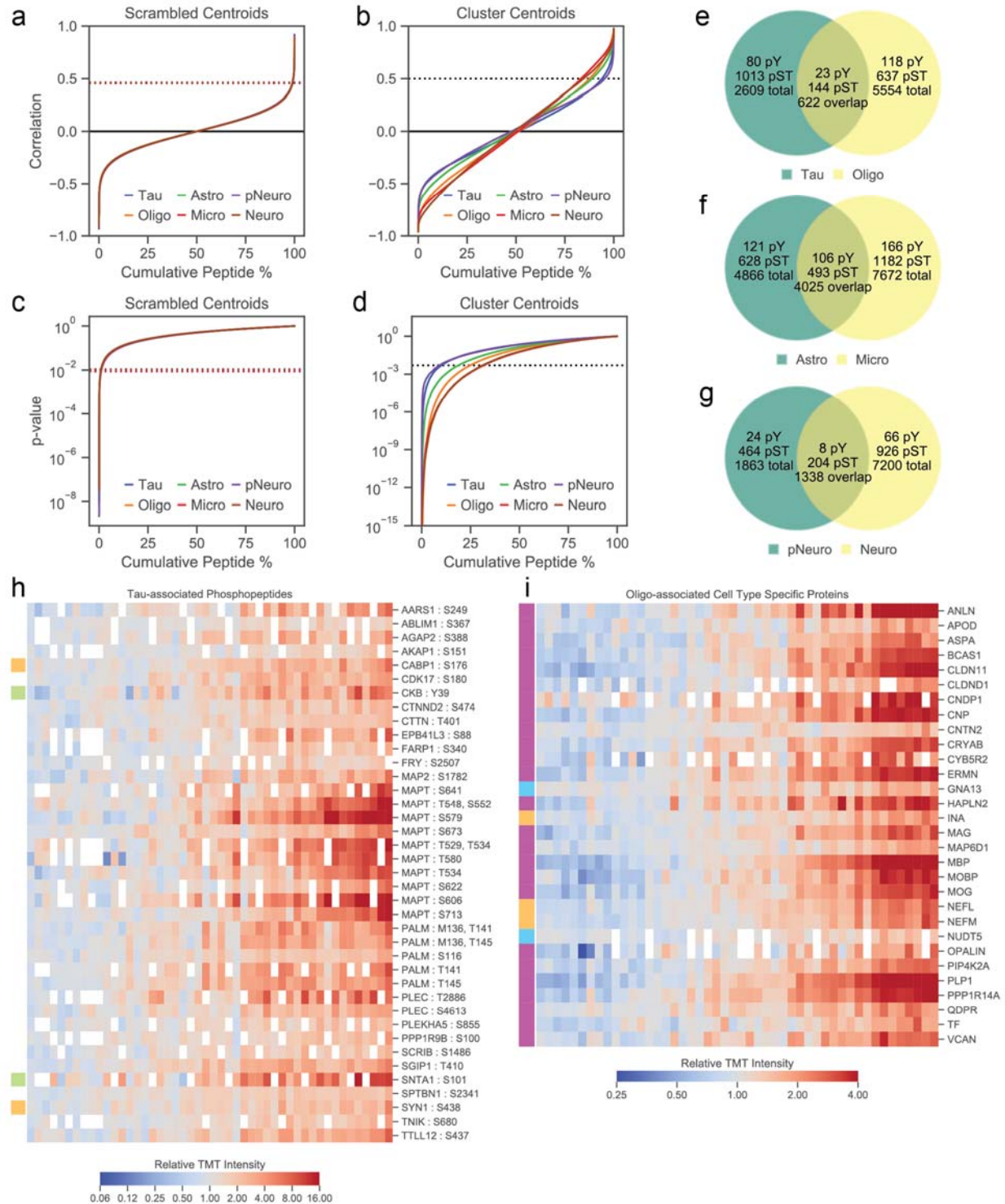
800

801 Supplementary Figure 3. **Peptide principle component analyses.**

802 (a) Principal component analysis (PCA) of peptides quantified in all samples. Patients are colored by (a)

803 AD or ND status, (b) Tau, (c) Oligo, (d) Astro, (e) Micro, (f) pNeuro, and (g) Neuro cluster centroid values.

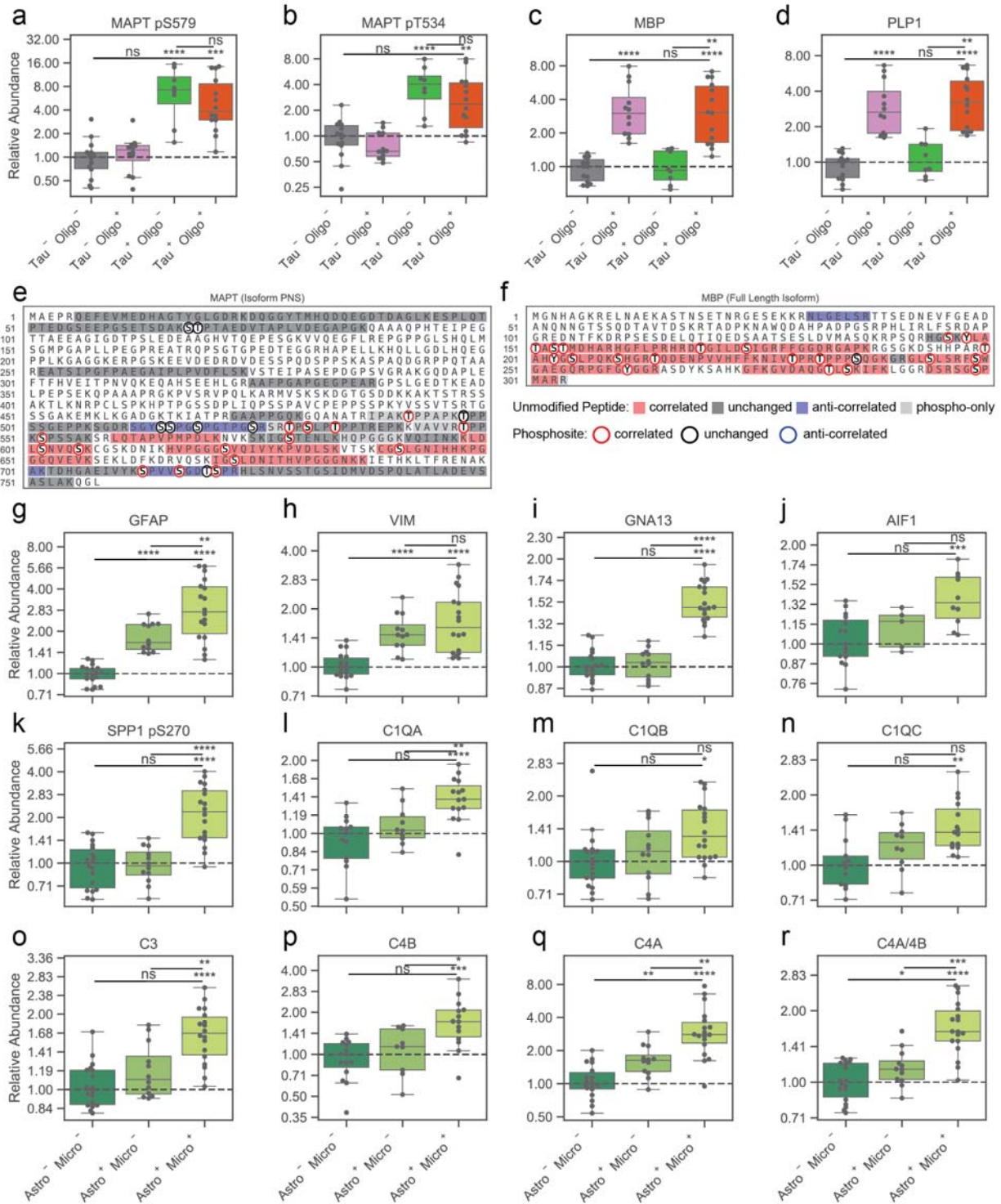
- 804 (h-j) PCA of samples colored by patient stratifications using (h) Tau and Oligo, (i) Astro and Micro, and (j)
805 pNeuro and Neuro cluster centroids.
- 806 (k-l) Patients numbers for (k) Astro;Micro or (l) pNeuro;Neuro patient stratifications.
- 807 (m-n) Overlap between Tau;Oligo and (m) Astro;Micro or (n) pNeuro;Neuro patient stratifications.



808
809 **Supplementary Figure 4. Peptide correlation analyses.**

810 (a-b) Distribution of Spearman's correlation coefficient for peptides identified in ≥ 15 patients with (a)
811 scrambled cluster centroids or (b) true cluster centroids.

- 812 (c-b) Distribution of Spearman's correlation p-values for peptides identified in ≥ 15 patients with (c)
813 scrambled cluster centroids or (d) true cluster centroids.
- 814 (e-g) Venn diagram showing the number of correlated peptides with (e) Tau and Oligo, (f) Astro and
815 Micro, and (g) pNeuro and Neuro cluster centroids. Plots include the total number of associated
816 peptides for each cluster, as well as the number of overlapping peptides between similar clusters.
- 817 (h) Heatmap showing phosphopeptide fold changes for peptides that are significantly correlated with
818 Tau cluster centroid. Samples are ordered by centroid values. Left color bars indicate proteins that were
819 predicted to be cell-type specific. Green = Astrocyte, Purple = Oligodendrocyte, Yellow = Neuron, Blue =
820 Microglia. Missing values are indicated as white boxes.
- 821 (i) Heatmap showing fold changes cell-type specific proteins that are significantly correlated with Oligo
822 cluster centroid. Samples are ordered by centroid value. Left color bar legend is same as (h).



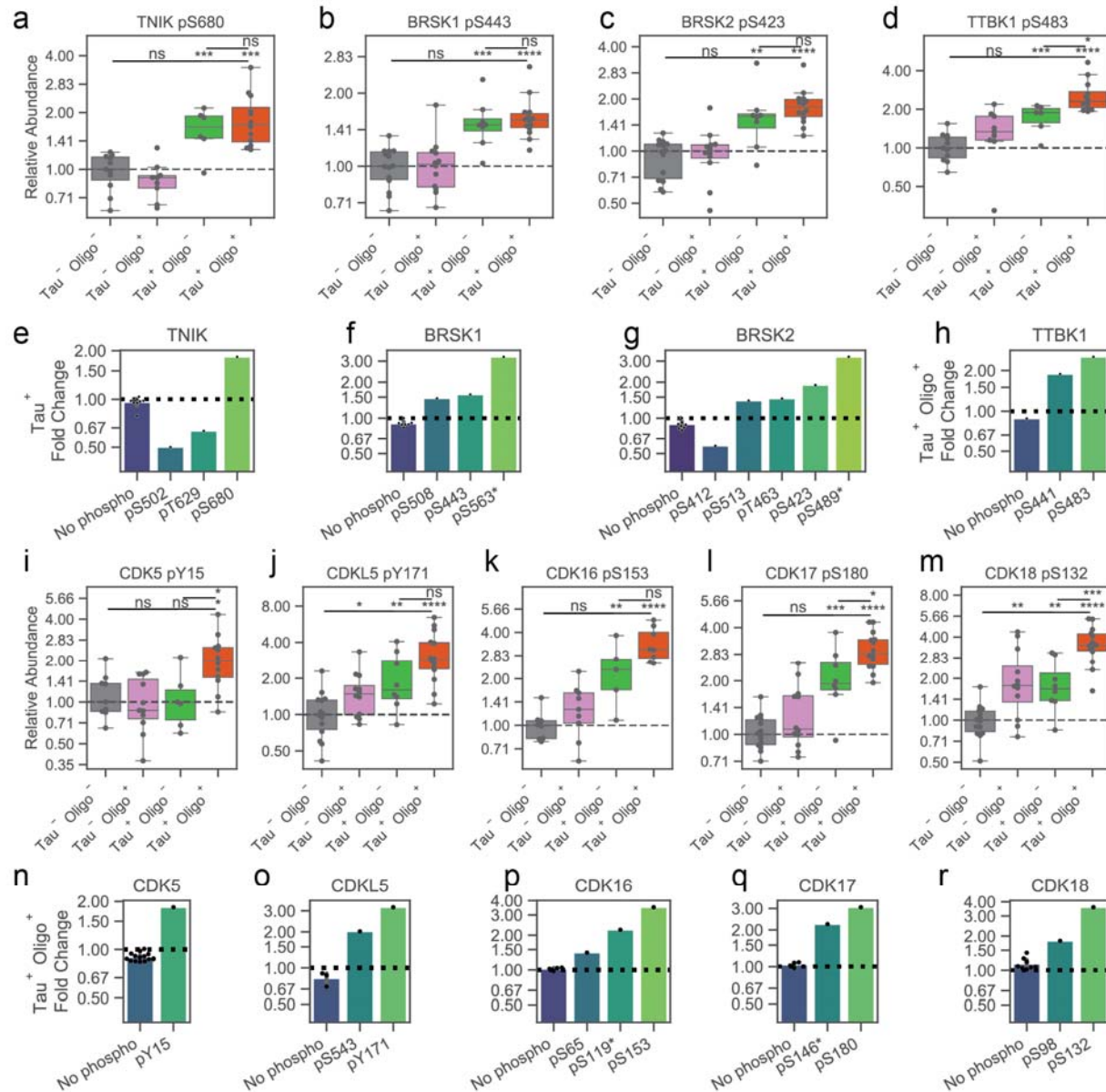
823
824 **Supplementary Figure 4. Analyses of peptide partial cluster correlation.**

825 (a-d) Fold changes for (a) MAPT pS579, (b) MAPT pT534, (c) MBP protein, and (d) PLP1 protein. Protein
 826 levels were estimated from the median abundance of all unmodified peptides that map uniquely to that
 827 protein. Values are normalized to the median of Tau⁻ Oligo⁻ patients. ns, not significant ($p > 0.05$); * $p <$
 828 $5e-2$, ** $p < 1e-2$; *** $p < 1e-3$; **** $p < 1e-4$.

829 (e) All peptides mapping to the peripheral nervous system (PNS) isoform of MAPT. Colored bars indicate
830 directional correlation for non-phosphorylated peptides. Red = correlated, blue = anti-correlated, grey =
831 uncorrelated, light-grey = only phosphopeptides were seen in that region. Colored circles indicate
832 phosphorylation sites that were quantified. Red circle = correlated, blue circle = anti-correlated, black
833 circle = uncorrelated.

834 (f) All peptides mapping to the full-length isoform of MBP. Legend is same as (e).

835 (g-l) Fold changes for (g) GFAP protein, (h) VIM protein, (i) GNA13 protein, (j) AIF1 protein, (k) ITGB2
836 protein, and (l) SPP1 pS270 across Astro and Micro groups



837

838 **Supplementary Figure 6. Tau kinase and CDK phosphosite abundances.**

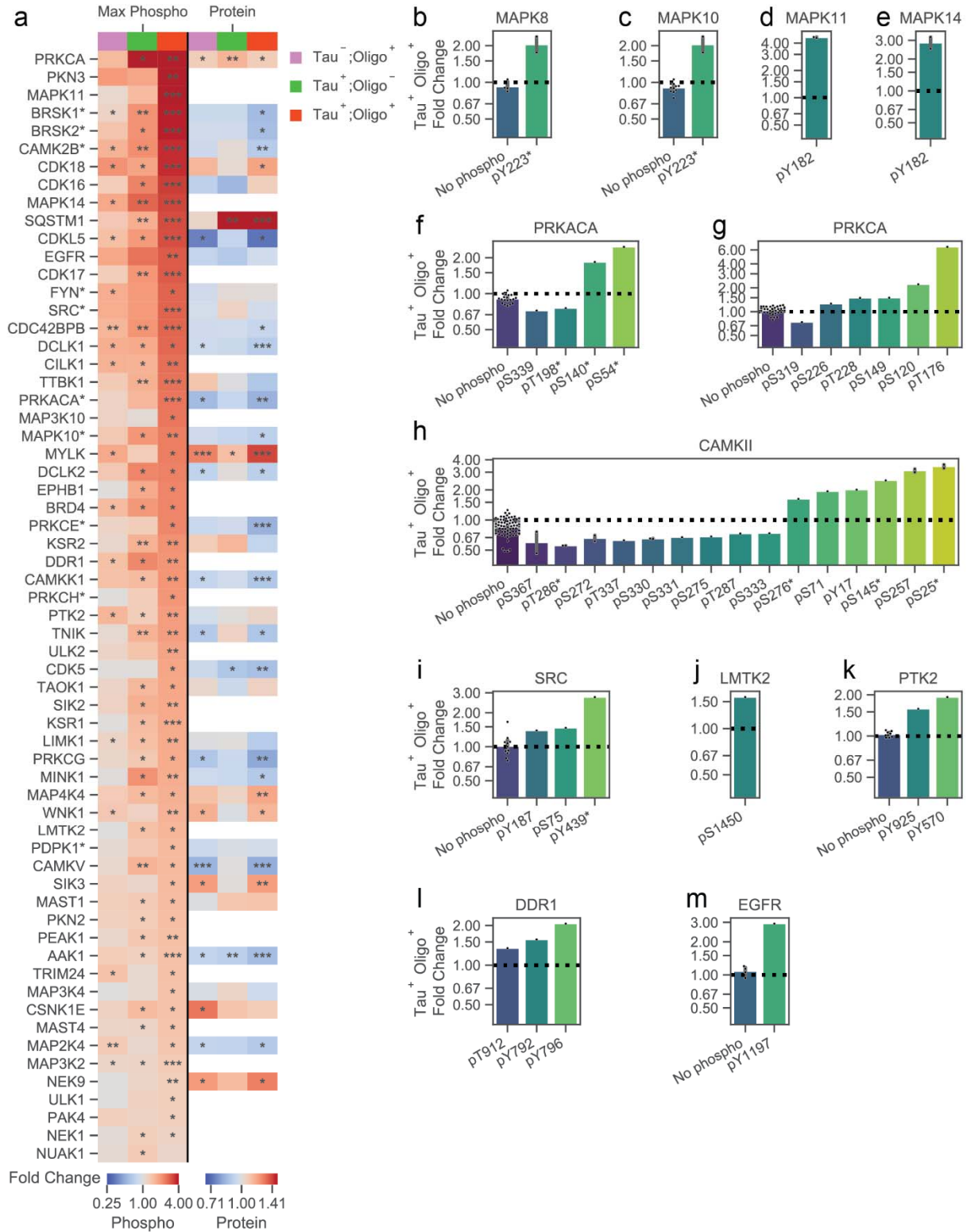
839 (a-d) Fold changes for (a) TNIK pS680, (b) BRSK1 pS443, (c) BRSK2 pS423, and (d) TTBK1 pS483 across
 840 Tau and Oligo groups.

841 (e-h) Fold changes for all non-phospho peptides and significantly changing phosphosites on (e) TNIK, (f)
 842 BRSK1, (g) BRSK2, (h) TTBK1. Each dot indicates one unique peptide. Phosphosites with '*' are on
 843 peptides that map ambiguously to more than one protein.

844 (i-m) Fold changes for (i) CDK5 pY15, (j) CDKL5 pY171, (k) CDK16 pS153, (l) CDK17 pS180, and (m) CDK18
 845 pS132 across Tau and Oligo groups.

846 (n-r) Fold changes for all non-phospho peptides and significantly changing phosphosites on (n) CDK5, (o)
 847 CDKL5, (p) CDK16, (q) CDK17, and (r) CDK18. Legend is same as (e-h).

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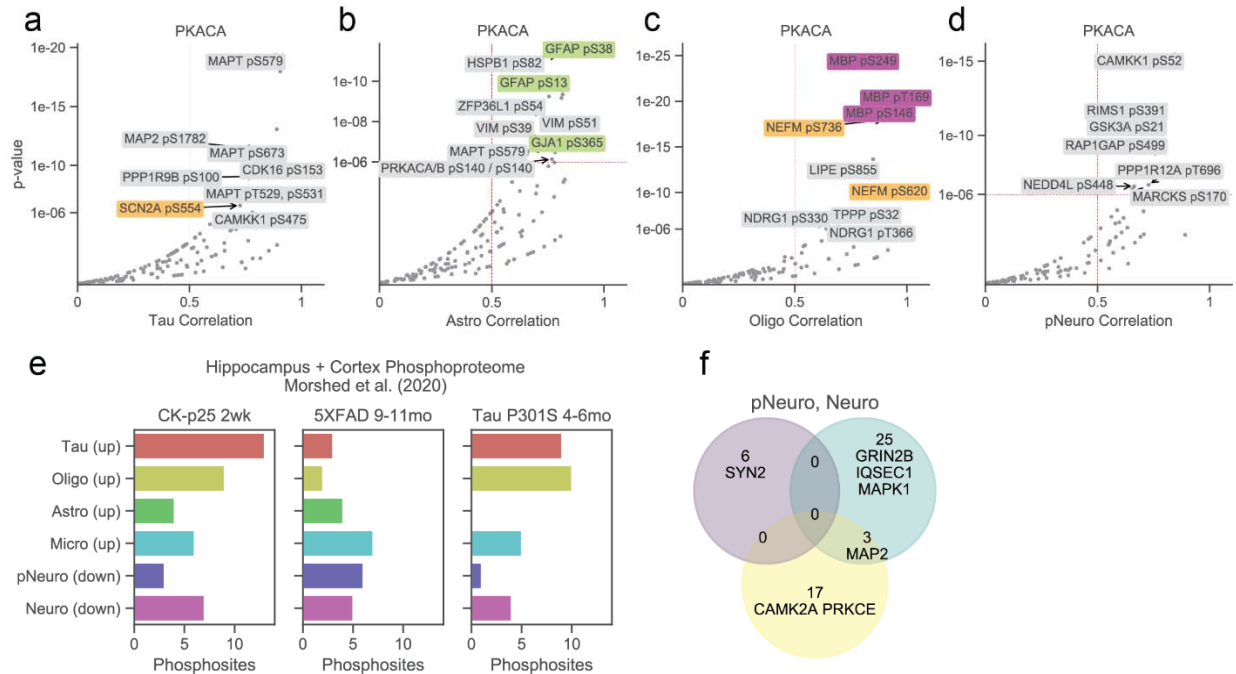


849

850 Supplementary Figure 7. Accessory kinome analyses.

851 (a) Maximum phosphosite fold changes and median protein abundances for all kinases shown in **Figure**
852 **4d**. Changes are calculated for each Tau;Oligo group compared to Tau;Oligo. *p < 5e-2; **p < 1e-3;
853 ***p < 1e-4.

854 (b-m) Fold changes for all non-phospho peptides and significantly changing phosphosites on (b) MAPK8
855 (c) MAPK10, (d) MAPK11, (e) MAPK14, (f) PRKACA, (g) PRKCA, (h) all CAMKII subunits, (i) SRC, (j) LMTK2,
856 (k) PTK2, (l) DDR1, and (m) EGFR. Legend is same as **Supplementary Figure 6e**.



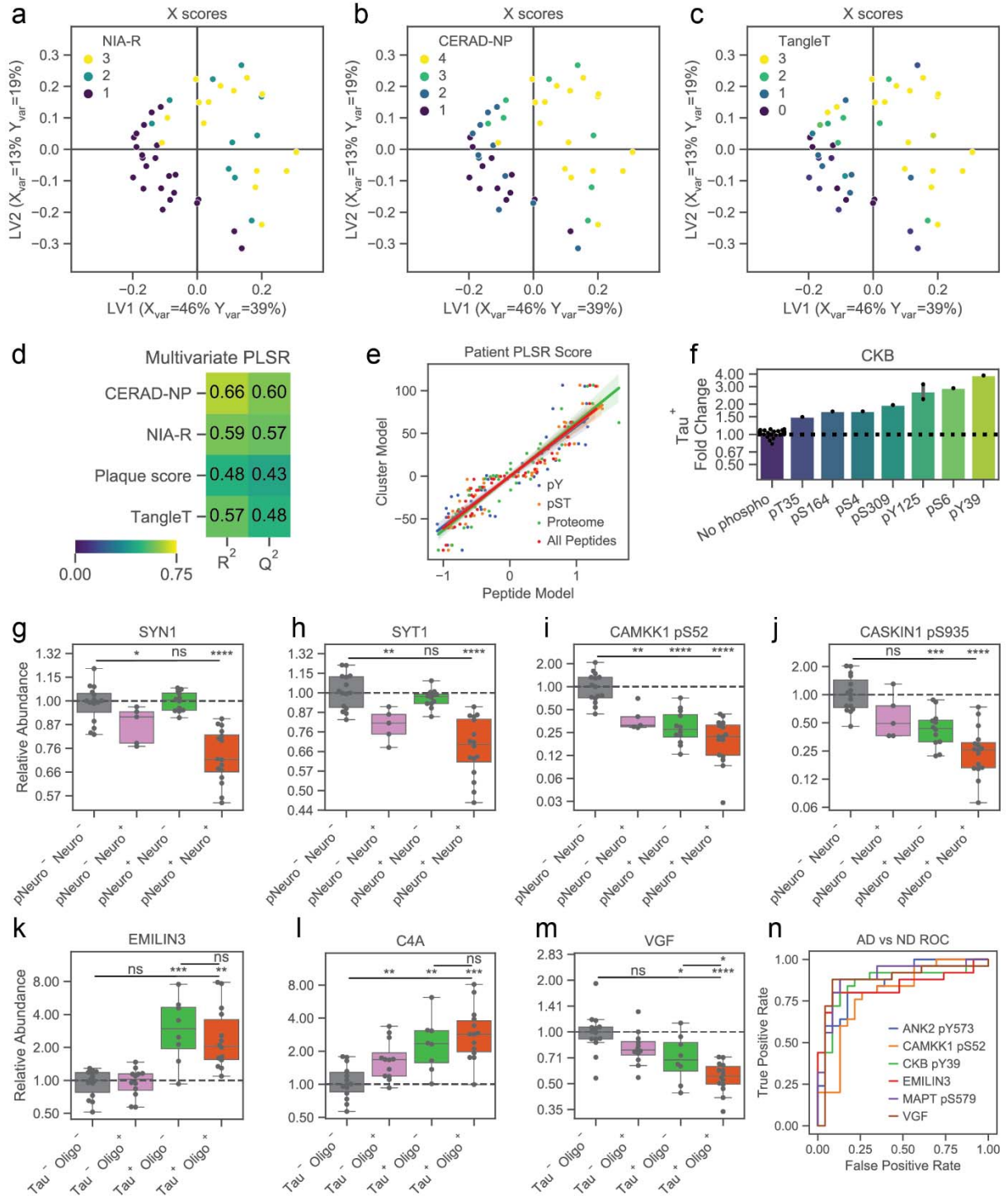
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858 **Supplementary Figure 8. Kinase-substrate and AD mouse model analyses.**

859 (a-d) Volcano plots showing Spearman's correlation coefficient and p-values between PKACA substrates and (a) Tau, (b) Astro, (d) Oligo, and (d) pNeuro cluster centroids. Labels are colored using the same
860
861 scheme as **Supplementary Figure 4h**.

862 (e) Number of phosphosites from AD mouse models identified in three mouse models of
863 neurodegeneration that could be translated to human and were significantly correlated with each
864 cluster centroid and shared directional changes.

865 (f) Phosphoproteins that were identified to have at least one shared phosphosite with three mouse
866 models of neurodegeneration. Proteins are shown as a Venn diagram between downregulated
867 phosphosites and pNeuro or Neuro cluster centroids.



868
869 **Supplementary Figure 9. PLSR accessory analyses.**

870 (a-c) Latent variable plots for multivariate PLSR analysis for X scores vectors. Points are colored by
871 patient (a) NIA-Reagan score, (B) CERAD-NP score, and (c) TangleT score.

872 (d) K-fold validation of the multivariate PLSR model for all included histopathology variables. R^2 and Q^2
873 values are shown for training and test data predictions respectively.

- 874 (e) Scatter plot showing PLSR patient scores generated from cluster centroids compared with subsets of
875 the matrix of peptides quantified in all patients.
- 876 (f) Fold changes for all non-phospho peptides and significantly changing phosphosites on CKB. Legend is
877 same as **Supplementary Figure 6e**.
- 878 (g-j) Fold changes for (g) SYN1, (h) SYT1, (i) CAMKK1 pS52, and (j) CASKIN pS935 across pNeuro and
879 Neuro groups.
- 880 (k-m) Fold changes for (k) EMILIN3, (l) C4A, and (m) VGF across Tau and Oligo groups.
- 881 (n) ROC curve predicting AD status from phosphosites and protein highlighted in **Figure 5f-5h**.

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