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## Unexpected Early Proteomic Changes in Alzheimer’s Disease Model Mice Synaptosomes

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## 31 **Abstract**

32           We have employed label-free quantitative proteomics of wild-type and Alzheimer's  
33 disease (AD) model mice synaptosomes to investigate proteomic changes occurring  
34 during AD progression as a prelude to analysis in humans. More than 4000 proteins were  
35 analyzed using multiple analysis tools and statistical criteria. Pathway enrichment  
36 identified numerous pathways consistent with the current AD knowledge base, including  
37 dysregulation of Glutamate Receptor Signaling, Synaptic Long Term Potentiation and  
38 Depression, Rho and Rac Signaling, Calcium Signaling, and Oxidative Phosphorylation  
39 and Mitochondrial Dysfunction. Additionally, the data demonstrate that a large number of  
40 changes occur in the proteome very early relative to the onset of both traditional disease  
41 markers such as amyloid accumulation, tau phosphorylation and cognitive dysfunction.  
42 These early changes include a number of dysregulated proteins that have novel  
43 associations with AD progression. These results reinforce the importance of mechanistic  
44 investigations in early disease progression long before the classical markers of  
45 Alzheimer's disease are observed.

## 46 **Introduction**

47           The synapse is the localized contact between nerve cells required for signal  
48 transmission and AD is considered by many to be a synaptic disease. This cell-to-cell  
49 communication is characterized by complex protein-driven molecular mechanisms  
50 including synthesis, delivery, storage, docking, fusion, neurotransmitter release and  
51 reuptake (1). Synapses can be studied by isolation of synaptosomes which contain the  
52 complete presynaptic terminal, including mitochondria and synaptic vesicles, along with

53 the postsynaptic membrane and the postsynaptic density. Several proteomic studies of  
54 synaptosomes have previously been performed (2–5). However, only recently has mass  
55 spectrometric analysis reached the level of technical advancement necessary for a direct  
56 and comprehensive analysis of the synaptic proteome (6). These advances in proteomics  
57 technologies allow direct and unbiased examination of protein level differences in  
58 neurodegenerative diseases and have great potential to shed new light on disease  
59 pathogenesis. Here, we employed these technical advancements in mass spectrometry  
60 for the detection of more than 4,000 synaptosomal proteins using label-free quantitative  
61 proteomics to characterize the proteome changes that occur in Alzheimer’s disease (AD)  
62 model mice. Multiple structural and/or metabolic proteins have been reported to have  
63 altered expression in AD supporting a high depth quantitative proteomic analysis for  
64 target discovery (7–10).

65

## 66 **Results**

### 67 **Mouse Model Characterization**

68 The B6C3-Tg(APP<sup>swe</sup>,PSEN1<sup>dE9</sup>)85Dbo/Mmjax mouse model (Tg-AD) of  
69 Alzheimer’s disease is a widely used model for AD; it contains a chimeric mouse/human  
70 amyloid precursor protein (APP)(Mo/HuAPP695<sup>swe</sup>) and human presenilin 1 (PS1-dE9);  
71 both driven by the prion protein promoter and therefore expressed in central nervous  
72 system neurons (11–15). These two insertions favor processing through the  $\beta$ -secretase  
73 pathway and, thus, elevate the amount of amyloid-beta ( $A\beta$ ) fragments produced from the  
74 APP transgene. To validate accumulation of  $A\beta$  fragments in transgenic mice, we used

75 commercially available ELISA kits specific for A $\beta$  1-42. As shown in Fig1, both A $\beta$  1-42  
76 accumulates in the brain of Tg-AD mice in an age dependent manner, supporting the  
77 choice of this mouse strain as a relevant model of AD.

78 **Fig1. Amyloid accumulation in the APP<sup>swe</sup>/PSEN1<sup>dE9</sup> Alzheimer's disease**  
79 **mouse model.** (A.) Human A $\beta$ 42 was quantitated using commercially available  
80 sandwich ELISA kits at the indicated ages using both wild-type (WT) and Tg-AD  
81 (B6C3-Tg (APP<sup>swe</sup>, PSEN1<sup>dE9</sup>)85Dbo/J) mice. Amounts of A $\beta$ 42 were  
82 normalized to total protein as determined by BCA Assay. (B.) Three, five and nine  
83 month old Tg-AD mice were chosen for high depth proteomic analysis based on  
84 the stages of A $\beta$  load, plaque development, and cognitive decline.

85 Based on our A $\beta$  accumulation data (Fig1) and upon previous characterizations of  
86 the APP<sup>swe</sup>, PSEN1<sup>dE9</sup> biogenic mouse model which describe the timing of cognitive  
87 impairment and plaque formation (11–15), we chose to carry out a proteomic analysis of  
88 the synaptosomes of three, five, and nine month-old mice. These three age groups  
89 represent three distinct stages of AD, summarized in Fig1C. The three month-old Tg-AD  
90 age group have minimal accumulation of A $\beta$ 1-42, normal cognitive function, and complete  
91 absence of plaques. The five month-old Tg-AD mice have relatively high levels of A $\beta$ 1-42  
92 which are accompanied by the presence of sporadic plaques although no cognitive  
93 decline has been reported. In contrast, the nine month-old Tg-AD mice represent the post-  
94 plaque and post-cognitive decline stage of AD.

95

## 96 **Workflow**

97           Synaptosomes from 3, 5, and 9 month-old Tg-AD and wild-type (WT) mice were  
98 isolated according to standard protocols (16). Filter Assisted Protein Preparation (FASP)  
99 was utilized to obtain pure peptides for LC-MS/MS analysis (17). Peptide samples were  
100 analyzed by online two dimensional reverse phase (RP/RP) nanoflow HPLC-MS/MS.  
101 Raw mass spectrometry data was converted to protein abundance using chromatography  
102 feature finding software MaxQuant (version 1.5.2.8) (18–20).

103           A total of 4,655 proteins were identified across all 18 samples using MaxQuant  
104 (18–20). All proteins categorized as potential contaminants, reverse sequence, and/or  
105 only identified by site were removed from the analysis. Biological replicates were then  
106 categorically annotated into 6 groups and proteins containing less than 2 valid values in  
107 each group were removed from the analysis, thus reducing the matrix to 3312 quantifiable  
108 proteins. (S1 Data File).

### 109           **S1 Data File. Protein Quantification and Analysis.**

110

## 111 **Qualitative Analysis**

112           A qualitative evaluation of the MS data was performed on both the WT and AD  
113 data. While biological variation is expected and accepted among replicates, due to  
114 complex and step-wise collection of RP/RP HPLC MS/MS data technical variations  
115 should be evaluated. For this analysis, we started with a hierarchical cluster analysis

116 (HCA) performed using complete-linkage clustering with Euclidean distance metrics of  
117 the nine WT samples (Fig2A) and the nine AD samples (Fig2B). As shown in Fig2A, the  
118 sample WT\_3M\_12f-C was identified as a potential outlier to other eight WT samples that  
119 are all merged into one cluster. The sub-clusters of the eight WT samples include pair-  
120 wise clustering of 3M with 5M and 5M with 9M, suggesting that age-dependent difference  
121 between the three WT age groups are negligible. In contrast, the main 2 clusters in the  
122 AD HCA (Fig2B) have a relatively short distance between them and the sub-clustering  
123 pairs include age matched AD samples, such as the pairing of AD\_3M\_14c-F with  
124 AD\_3M\_12f-AD, AD\_9M\_16c-CF with AD\_9M\_16a-D, and AD\_5M\_11a-C with  
125 AD\_5M\_11a-AB. Thus, the AD HCA suggests that differences between the age groups  
126 and, moreover, differences between the three selected stages of Alzheimer's disease will  
127 be observed.

128 **Fig2. Qualitative Assessment of MS/MS data.** A dendrogram of the WT samples  
129 (A.) and the AD samples (B.) produced using Euclidean distance with complete  
130 linkage of LFQ intensity data with all invalid values removed. (B.) Histogram of  
131 absolute  $\log_2$  expression values (ORANGE:  $WT_x - WT_{average}$ , GREEN:  
132  $3M_x - 3M_{average}$ , BLUE:  $5M_x - 5M_{average}$ , YELLOW:  $9M_x -$   
133  $9M_{average}$ ).

134  
135 To further explore the variability between replicates, we evaluated the  $\log_2$  fold  
136 change of individual samples from the average (Fig2C, ORANGE: WT from the average  
137 WT, GREEN: 3M\_AD from the average 3m Tg-AD, BLUE: 5M\_AD from the average 5m  
138 Tg-AD, and YELLOW: 9M\_AD from the average 9m Tg-AD). The expectation is that all

139 samples included in the average should have a similar shaped distribution, thus the  
140 shorter and broader shape of the sample WT\_3M\_12f-C distribution compared to the  
141 other WT samples further supports the identification of this sample as an outlier. The  
142 3M\_AD samples all have a similar distribution, despite the Euclidean distance of sample  
143 AD\_3M\_14c-A from the other two 3M\_AD samples (Fig2B). While the AD\_5M and  
144 AD\_9M sample distributions are a clear reflection the short Euclidean distances observed  
145 in Fig2B; in particular, the distance between AD\_5M\_11a-C and AD\_5M\_11a-AB is  
146 shorter than for any other pairing and the distribution of these samples is taller and more  
147 narrow than any of the other samples.

148 Together, this qualitative evaluation of the MS/MS data identifies sample  
149 WT\_3M\_12f-C as an outlier that will be excluded from the quantitative analysis.  
150 Additionally, this analysis supports that age dependent biological variability between the  
151 control groups is very small. Grouping the 8 WT samples in one control group increases  
152 the statistical power of the analysis, but may sacrifice age specific variations, thus further  
153 evaluation of age dependent protein abundance was analyzed.

154 To determine if pooling the WT samples would be appropriate, we analyzed  
155 potential age-dependent changes in the WT samples using an ANOVA multi-sample test.  
156 No statistically significant proteins were identified using a loose False Discover Filter  
157 (FDR, Bengamini-Hochburg)  $\alpha = 0.25$ . Thus, to identify genes that would likely lose  
158 statistical relevance if an average WT was used rather than age matched controls, we  
159 applied a p-Value cut-off of 0.1 with no FDR and an absolute  $\log_2$  fold change filter of 0.5.  
160 We identified 73 proteins of interest (S1 Data File) that could be lost if we pooled the WT

161 samples into a single control group. However, despite these potential losses, we made the  
162 decision to group all of the WT samples into a single control group.

163

## 164 **Quantitative Assessment of Alzheimer's Disease Progression Proteome**

165 R-limma (21–23) was used to perform a quantitative assessment of proteome  
166 differences between the following groups: 3m Tg-AD & WT, 5m Tg-AD & WT, and 9m Tg-  
167 AD & WT. Empirical Bayes statistics was used to calculate p-Values. Using a Benjamini-  
168 Hochburg FDR  $\alpha = 0.1$ , (Fig3; S1 Data File). Surprisingly, the majority of statistically  
169 significant proteins identified were only significant in the pre-plaque, 3m Tg-AD, stage of  
170 AD when A $\beta$  levels are still very low. The protein expression profiles of the top most  
171 dysregulated proteins ( $\log_2$  FC  $\geq |1|$ ) are shown in Fig4. We noted that even though the  
172 selection of significant proteins was dominated by the 3m Tg-AD statistical analysis, clear  
173 age dependent data trends were observed; we clustered the proteins based on these  
174 expression trends. This clustering shows groups of proteins that decrease (clusters 2 &  
175 3) and increase (clusters 8, 9, & 10) during AD progression, that are consistently up  
176 (cluster 1 & 2) or down (cluster 10 & 11), and that have stage specific protein  
177 dysregulation (clusters 4, 5, 6, & 7). Eleven of the proteins of interest identified have a  
178 previous association with A $\beta$  and/or AD (Fig4 \*GENE), while 73 of these high confidence  
179 proteins ( $\log_2$  FC  $\geq |1|$ ) are novel to our understanding of AD progression.

180 **Fig3. Quantitative Analysis of Age-Dependent Alzheimer's Disease.** R-limma  
181 was used to calculate the p-Value and  $\log_2$  fold change for the 3312 quantifiable  
182 proteins using the following groupings: (A.) 3m Tg-AD & WT, (B.) 5m Tg-AD & WT,



183 and (C.) 9m Tg-AD & WT. A Benjamini-Hochburg FDR  $\alpha = 0.1$  is indicated with a  
184 solid line in each graph and the significant proteins are colored. This data and the  
185 corresponding gene names are included in Supplement File 1.

186

187 **Fig4. High-Confidence Protein Expression Profiles and Clusters.** Proteins  
188 were filtered using an FDR = 0.1 and an absolute FC = 1 in at least one age group.  
189 Proteins were clustered based on observable FC trend. Proteins with a previously  
190 recognized association with A $\beta$  (24) and/or recognized by Kegg as “Alzheimer’s  
191 Disease: are indicated by \* next to the gene name.

192

## 193 **STEM Analysis**

194 As mentioned above, we observed many proteins that appeared to have a linear  
195 trend of protein expression. Time-dependent data trends can further increase confidence  
196 in the data, and analysis of these trends may even identify additional statistically significant  
197 protein changes. To further explore proteins with trending protein profiles we utilized the  
198 Short Time-series Expression Miner (STEM)(25). We used a maximum of 50 model  
199 profiles and maximum unit change of 3 ( $\log_2$  FC) between time points to profile the matrix  
200 of 3312 quantifiable proteins at the three Tg-AD age groups. STEM profile enrichment  
201 identified 121 proteins that follow one of five significant profiles (S2 Data File). Fig5A-E  
202 shows these significant STEM profiles (black); the protein expression patterns that were  
203 fitted to these profiles are plotted along with the STEM profiles. Not quite half the proteins  
204 identified by STEM (58/121 proteins) were identified as significant using the empirical

205 Bayes statistical enrichment, while 63 new proteins were added to the list of proteins  
206 dysregulated during the progression of AD.

## 207 **S2 Data File. Time-series expression analysis by STEM.**

208 **Fig5. Significant Protein Trends identified by STEM.** Five protein expression  
209 profiles were identified as having more proteins fitting to the profile than would be  
210 expected by random chance. These significant profiles included three increasing  
211 profiles with 41 proteins fitting the -3, 0, 1 log<sub>2</sub> FC profile (A.) in the 3m, 5m, and  
212 9m age groups, respectively, 25 proteins fitting the -1, 0, 3 log<sub>2</sub> FC profile (B.), and  
213 16 proteins fitting the -2, -1, 1 log<sub>2</sub> FC profile (C.). Two decreasing profiles were  
214 also identified; these were characterized by 25 proteins fitting the 3, 0, -1 log<sub>2</sub> FC  
215 profile (D.) and 14 proteins fitting the 2, 1, -1 profile (E.) The profile summaries  
216 exported from STEM can be found in S2 Data File and the protein-to-profile  
217 assignments can be found in S2 Data File as well as in S1 Data File.

218

## 219 **Canonical Pathway Enrichment**

220 The benefit of adding proteins identified by STEM is demonstrated in Fig6 where  
221 Canonical Pathway Enrichment (by Ingenuity Pathway Analysis, IPA) is compared  
222 between three data filters for the 3m Tg-AD data set: a strict filter (FDR 0.1 + log<sub>2</sub> FC >=  
223 |1|), an FDR only filter (FDR 0.1), and a STEM enriched filtered data set (FDR 0.1 + log<sub>2</sub>  
224 FC >= |0.4|, +STEM proteins) for a select group of canonical pathways (complete list: S3  
225 Data File). P-values for canonical pathways are calculated in IPA using a right-tailed  
226 Fisher Exact Test that considers the overlap of observed and predicted genes in a

227 pathway. Thus, while strict data filters will result in a high confidence gene list (Fig4), a  
228 short gene list will also produce a low confidence canonical pathway enrichment.  
229 Additionally, Fig6 & S3 Data File show that, with few exceptions, expanding the gene list  
230 to include lower confidence protein expressions identified using STEM adds to our  
231 pathway confidence (p-Value) or in other words, “what is significant becomes MORE  
232 significant”. IPA also assigns a Z-score that assesses the match of observed and  
233 predicted up/down regulation patterns. Therefore, the age-dependent FC for all proteins  
234 in the Canonical Pathway are considered. In IPA, a Z-score greater than 2 or less than -  
235 2 is considered predictive; positive Z-scores indicate activation and negative Z-scores  
236 indicate repression of the described function. As shown in Fig6, the STEM enriched  
237 protein list allows higher confidence directional predictions in comparison the FDR only  
238 filtered protein list. No predictable directionality was found in any of the enriched  
239 Canonical Pathways when the high confidence protein list (FDR 0.1, |FC|>1) was used.

### 240 **S3 Data File. Canonical Pathway Enrichment.**

241 **Fig6. STEM proteins add confidence and direction to Canonical Pathway**  
242 **Enrichment analysis.** Ingenuity Pathway Analysis (IPA) was used to analyze the  
243 3m Tg-AD Canonical Pathway Enrichment using three different data filters: the  
244 high confidence protein list from Fig4, a standard filter (FDR = 0.1 only), and the  
245 STEM expanded FDR = 0.1 and absolute FC  $\geq$  0.4. p-Values and z-scores as  
246 calculated by IPA are shown for the top Canonical Pathways. The complete  
247 Canonical Pathway Enrichment list can be found in S3 Data File.

248

249 With this in mind we utilized the protein expression data from all proteins identified  
250 as significant (FDR = 0.1, absolute  $\log_2$  FC  $\geq$  0.4) plus the STEM identified proteins to  
251 run a canonical pathway enrichment analysis. All pathways with a z-score in at least one  
252 age group are shown in Fig7. Z-scores greater than or equal to the absolute value of two  
253 are considered predictive. All dysregulated pathways, including those without a z-score,  
254 can be found in S3 Data File.

255 **Fig7. Ingenuity Pathway Analysis (IPA) predicted direction of Canonical**  
256 **Pathways.** IPA was used to analyze compare the directionality (z-scores) of  
257 Canonical Pathways identified enriched in the STEM expanded FDR = 0.1 and  
258 absolute FC  $\geq$  0.4 protein list. Canonical pathways with valid/non-zero z-score in  
259 at least one age group are shown here. The complete Canonical Pathway  
260 Enrichment list can be found in S3 Data File.

## 261 Discussion

262 The most well-known and well-studied histopathological hallmark of AD is an  
263 increase in A $\beta$  peptide abundance and subsequent formation of amyloid plaques.  
264 Accordingly, the mouse model used in our studies should provide insights in downstream  
265 mechanisms following A $\beta$  production. A $\beta$  is believed to be a crucial pathogenic factor in  
266 AD development. Recent evidence indicates that the soluble-oligomeric forms of A $\beta$  are  
267 primarily responsible for the neurodegeneration and loss of synaptic function  
268 characteristic of later stages of AD, and this soluble-A $\beta$  hypothesis is further supported  
269 by recent clinical data on aducanumab, a human monoclonal antibody shown to reduce  
270 soluble and insoluble A $\beta$  (26).

271 Surprisingly, we observed that the largest changes to the synaptosomal proteome  
272 occurred in the 3m Tg-AD mouse model where A $\beta$  levels are relatively low, A $\beta$  plaques  
273 are absent, and no cognitive decline is observable. While this is consistent with soluble-  
274 oligomeric forms of A $\beta$  being primarily responsible for the pathogenesis of AD, it is notable  
275 that despite the large proteomic changes, the 3m Tg-AD mice have no observable  
276 phenotype. Very recently, a new AD phenotype has been observed: olfactory recall  
277 impairment occurs up to ten years prior to the onset of cognitive decline (27,28). This  
278 suggests that the observed proteomic changes in the 3m Tg-AD mouse may have  
279 correlated phenotypes that remain to be identified. However, it could also suggest that A $\beta$   
280 has a significant biological impact on the brain, even at low levels, and that compensatory  
281 mechanisms are not only active but, most importantly, are biological efficacious during  
282 the preclinical stages of AD. Although the ultimate progression of AD suggests that any  
283 early neuroprotective response to A $\beta$  is not sustainable or is not sufficient to prevent  
284 neurotoxicity.

285 In typical AD progression, A $\beta$  promotes disturbances in a number of pathways that  
286 ultimately lead to neurotoxicity. Specifically, A $\beta$  oligomers have been reported to induce  
287 NMDA receptor activation, mitochondrial Ca<sup>2+</sup> overload/membrane depolarization,  
288 oxidative stress and apoptotic cell death (29–32). Consistent with this pathology, we  
289 observed dysregulation in these Canonical Pathways: Glutamate Receptor Signaling,  
290 Synaptic Long Term Potentiation and Depression, Calcium Signaling, and Oxidative  
291 Phosphorylation and Mitochondrial Dysfunction (Figures 6 & 7, S3 Data File). Beyond  
292 observing dysregulation of Canonical Pathways that are consistent with what is already  
293 known about AD, we identified 73 proteins with high confidence ( $\log_2$  FC  $\geq$  |1|, Fig4) that

294 are novel in our understanding of AD progression, but that are consistent with previous  
295 studies. While refraining from going through all proteins/pathways, a striking example of  
296 how our data supports the existing knowledge can be found by close inspection of the  
297 RhoA Pathway depicted in Fig8. Specifically, our data shows an increase RhoA GEF  
298 protein (ARHGEF1) and a decrease in RhoA GAP protein (RHOGAP) in the 3m Tg-AD  
299 (Fig8A), consistent with activation of RhoA Signaling Pathway (Fig8B). RhoA activation,  
300 as shown in Fig8A & B, leads to the activation a number of kinases whose downstream  
301 activities regulate the actin cytoskeleton. ROCK, for example, is a kinase downstream of  
302 RhoA that activates LIMK which in turn phosphorylates, and thus inhibits, the actin  
303 severing protein, cofilin (Fig8). However, in direct opposition to the IPA's predicted state  
304 of cofilin, previous observations with A $\beta$ 1-42 treatment show an increase in  
305 dephosphorylated cofilin (33) and an increase of cofilin translocation into the mitochondria  
306 (consistent with dephosphorylated cofilin) (34). Moreover, in AD patients, a loss or  
307 shortening of dendritic spines is observed, consistent with loss of cytoskeletal stability.  
308 Previous studies have also shown that A $\beta$ 1-42 treatment of hippocampal neurons induced  
309 increased activity in Rac1, Cdc42, and PAK1 (33), which, like RhoA, are also involved in  
310 activation of LIMK and the phosphorylation of cofilin. Together, this data suggests that A $\beta$   
311 either inhibits phosphorylation or promotes dephosphorylation of cofilin and that activation  
312 of upstream activators of LIMK may be a compensation mechanism for the increase in  
313 dephosphorylated cofilin. Potential mechanisms of A $\beta$ 's regulatory role on cofilin  
314 phosphorylation and dephosphorylation are illustrated in Fig9A.

315 **Fig8. RhoA Signaling Pathway activation in early AD.** (A.) Canonical Pathway  
316 enrichment analysis identified RhoA as activated in the 3m Tg-AD. (B.) The log<sub>2</sub>

317 FC of eight proteins within the RhoA Signaling Pathway were used to calculate the  
318 z-score and the predicted directionality of RhoA Signaling. (C.) These molecules  
319 are colored red (up-regulated) or blue (down-regulated) in a schematic of the RhoA  
320 Signaling pathway in the 3m Tg-AD. (D.) IPA's Molecular Activation Predictor  
321 illustrates the predicted outcome of the 3m Tg-AD protein abundance changes.  
322 (Red = activated; Blue = inhibited; Yellow = inconsistent with state of downstream  
323 molecule)

324 **Fig9. Proposed A $\beta$ 's impact on Cofilin Phosphorylation and actin**  
325 **stabilization.** Our study, in concert with previous studies, implicates A $\beta$   
326 involvement in the activation of a number of molecules upstream of Cofilin  
327 regulation and actin stabilization. (Red = activated; Blue = inhibited; Yellow =  
328 inconsistent with state of downstream molecule)

329  
330 Compensation for A $\beta$  induced dysregulation of cofilin-actin dynamics would be  
331 required for sustained neural function and survival. Cofilin-actin regulation is critical for  
332 morphogenesis and the structural dynamics of neural spines and has been strongly  
333 implicated in synaptic trafficking of AMPA receptors during Synaptic Potentiation and  
334 Depression (35,36). In the 3m Tg-AD, we do not see a significant difference in the number  
335 of AMPA receptors (GRIA). However, we assume there is successful compensatory  
336 stabilization of actin filaments through the RhoA Signaling Pathway.

337 The RhoA Signaling Pathway represents only one of many pathways that were  
338 found to be dysregulated in the pre-clinical 3m Tg-AD model. Understanding how these

339 pathways take part in the response to A $\beta$  may lead to new therapeutic avenues for AD.  
340 Additionally, recognition of the early dysregulation of these pathways may help identify  
341 new, pre-clinical, phenotypes for AD.

## 342 **Methods**

### 343 **Mice**

344 Mice used in the experiments were housed in accordance with protocols approved  
345 by the Institutional Animal Care and Use Committee at University of Colorado and all  
346 experiments were conducted according to the NIH Guide for the Care and Use of  
347 Laboratory Animals. The B6C3-Tg(APP<sup>swe</sup>, PSEN1<sup>dE9</sup>)85Dbo/J breeding pair was  
348 obtained from The Jackson Laboratory (stock number 004462). Colony was maintained  
349 using +/+ sibling x Hemi zygote. Ear punches were taken at approximately 10 days old  
350 and PCR identification was performed to identify AD transgenic mice and the non-  
351 transgenic littermate controls. The AD transgenic mice and the non-transgenic littermate  
352 controls of the same sex were housed in individual ventilated cages, with a maximum of  
353 five mice per cage. Female B6C3-Tg(APP<sup>swe</sup>, PSEN1<sup>dE9</sup>)85Dbo/J transgenic mice at  
354 various ages (3, 5, and 9 months) and their age-matched, non-transgenic littermate  
355 controls (wild type, WT) were used in this study. For quality control purposes tail clips  
356 were taken at the time of death and a second genotyping was performed to confirm the  
357 first.

### 358 **ELISA**

359 Hemi-brain samples were analyzed for human A $\beta$  1-40 and 1-42 using  
360 commercially available ELISA kits (Life Technologies: KHB3481 & KHB3441) according



361 to manufacturer's instruction. Amounts of A $\beta$  were normalized to total protein as  
362 determined by BCA assay (Pierce #23225).

## 363 **Synaptosome Isolation**

364 All mice were sacrificed at the age indicated. Synaptosomes were isolated as  
365 previously described with minor modifications (16). The whole brain was dissected from  
366 one mouse and homogenized with 20 strokes in 2 mL of complete sucrose buffer (0.32  
367 M Sucrose; 2 mM EGTA; 2 mM EDTA; 10 mM HEPES (pH 7.4), 1x Protease Inhibitor  
368 Cocktail: cOmplete, EDTA-free (Roche 05 056 489 001), 1mM Na<sub>3</sub>VO<sub>4</sub> (Sodium  
369 Vanadate), 1mM Na<sub>4</sub>O<sub>7</sub>P<sub>2</sub> (Sodium Pyrophosphate). Centrifuged at 800 x g for 10 minutes  
370 at 4°C to pellet the membrane fragments and nuclei and collect supernatant (S1) in 15  
371 mL conical tubes then fast frozen in liquid nitrogen and then stored at -70°C until all mouse  
372 brain samples for were collected. To minimize technical variability, synaptosomal  
373 preparation was performed in age-matched batches. All batch purifications were  
374 performed with the same stock buffers within 24 hours of each other. To prepare purified  
375 synaptosomes the S1 samples were thawed on ice, centrifuged at 800 x g for 10 minutes  
376 at 4°C and collect supernatant (S1') in high-speed polycarbonate tubes. S1' fractions  
377 were then centrifuged at 10,000 x g for 15 minutes at 4°C to obtain a pellet (P2) containing  
378 synaptosomes contaminated with mitochondria and microsomes. P2 was suspended in  
379 500  $\mu$ l of Complete Sucrose Buffer, vortexed thoroughly then loaded on a sucrose  
380 gradient (from bottom to top): 1.18 M – 1.0 M – 0.85 M (all prepared in 10 mM HEPES,  
381 pH 7.4, 2 mM EDTA, 2 mM EGTA) prior to centrifuging at 82,500 x g for 1 hour at 4°C.  
382 Pure synaptosomes were collected from the interface between 1.0 M and 1.18 M, washed  
383 by adding ~3X volume of Complete Sucrose Buffer and centrifuged at 10,000 x g for 15

384 minutes at 4°C prior to re-suspending in Complete Sucrose Buffer and measuring of  
385 protein concentration using a micro BCA Protein Assay (Pierce 22660) according to  
386 manufacturer's instructions. Pure synaptosomes were divided into 50 µg aliquots and  
387 stored at -80°C.

## 388 **Peptide Preparation**

389 Peptide preparation was performed by the Filter Assisted Sample Preparation  
390 (FASP) method as described (17). Briefly, to extract membrane proteins in the  
391 synaptosome samples, a 0.3% concentration of Triton X-100 was used to solubilize 50  
392 µg of purified synaptosomes. The sample was then washed with fresh 8 M and 2 M Urea  
393 Buffer in a 30 kDa filter (Millipore UFC 503024); the proteins were reduced (10 mM TCEP)  
394 and alkylated (25 mM IA) and treated with trypsin (Promega #V5113; 1:100 or 0.05 µg of  
395 trypsin per 50 µg sample) overnight in the spin filter. The resulting peptides were desalted  
396 in C-18 spin columns (Pierce 89870) according to the manufacturer's instructions. The  
397 peptide concentration was estimated using a NanoDrop 2000 and Bovine Serum Albumin  
398 as reference (mass extinction coefficient of 6.7 at 280 nm) resulting in 6-7 µg of peptide  
399 per sample. The peptide was then immediately lyophilized for 2 hours, and stored at -  
400 80°C.

## 401 **RP-RP MS/MS**

402 The peptides were separated by liquid chromatography using a nanoAcquity UPLC  
403 system (Waters) coupled to a LTQ Orbitrap mass spectrometer (Thermo Fisher  
404 Scientific). Peptide mixtures (2 µg) were loaded onto a 300-µm × 50-mm XBridge C18,  
405 130-Å, 5-µm column maintained at pH 10.0, eluting peptides in six fractions

406 corresponding to 10%, 15%, 20%, 30%, 40%, and 60% buffer B1 (buffer A1: 20 mM  
407 ammonium formate, pH 10.0; and buffer B1: 100% acetonitrile). Steps were eluted from  
408 the high pH column at 20  $\mu$ l/min onto a 180- $\mu$ m  $\times$  20-mm C18, 100-Å, 5- $\mu$ m trap column,  
409 which was then switched in-line with the analytical column and eluted as in the 1D  
410 method. For 1D analysis, a BEH C18 reversed phase column (25 cm  $\times$  75  $\mu$ m i.d., 1.7  
411  $\mu$ m, 100 Å; Waters) was used for the analytical separation using a linear gradient from  
412 90% buffer A2 (0.1% formic acid) to 40% buffer B2 (0.1% formic acid and 80%  
413 acetonitrile) over 60 min at a flow rate of 300 nL/min.

414 MS/MS data were collected by an enabling monoisotopic precursor and charge  
415 selection settings. Ions with unassigned charge state were excluded. For each mass  
416 spectrometry scan, the 10 most intense ions were targeted with dynamic exclusion 30 s,  
417 1 D exclusion width, and repeat count equal to 1. The maximum injection time for Orbitrap  
418 parent scans was 500 ms, allowing 1 microscan and automatic gain control of 106. The  
419 maximal injection time for the LTQ MS/MS was 250 ms, with 1 microscan and automatic  
420 gain control of 104. The normalized collision energy was 35%, with activation Q of 0.25  
421 for 30 ms.

## 422 **Data Analysis**

423 The raw MS/MS data from all samples were analyzed by MaxQuant (37)(version  
424 1.5.2.8). Andromeda (38), a probabilistic search engine incorporated into the MaxQuant  
425 framework was used to search the peak list against the Uniprot\_MOUSE database  
426 (UniProtKB release 2016\_098, entries: 82,200). Common contaminants were added to  
427 this database. The search included cysteine carbamidomethylation as a fixed modification

428 and N-terminal acetylation and methionine oxidation as variable modifications. The false  
429 discovery rate (FDR) was set to 0.01 for both peptide and protein identifications. Enzyme  
430 specificity was set to trypsin allowing N-terminal cleavage to proline. Two miscleavages  
431 were allowed, and a minimum of seven amino acids per identified peptide were required.  
432 Peptide identification was based on a search with an initial mass deviation of the  
433 precursor ion (ITMS) of up to 0.5 Da, and the allowed fragment mass deviation (FTMS)  
434 was set to 20 ppm. Razor peptides were used for quantification; unmodified or with the  
435 modifications specified above. To match identifications across different replicates and  
436 adjacent fractions, the “match between runs” option in MaxQuant was enabled within a  
437 matching time window of 0.7 min.

438 Bioinformatics analysis was done with Perseus (39)(version 1.4.1.3) tools  
439 available in the MaxQuant environment. The proteins only identified by site, from the  
440 reverse database, and contaminant proteins were removed from the matrix. Categorical  
441 annotation by age and type was performed resulting in 6 groups: AD\_3M, AD\_5M,  
442 AD\_9M, WT\_3M, WT\_5M, & WT\_9M, with an n=3 in each group. The protein matrix was  
443 reduced to those identifications with at least one valid observation in each group. A  
444 multiple-sample test was run between WT\_3M, WT\_5M, & WT\_9M using a Bengamini-  
445 Hochburg FDR=0.25 (7 significant) and these proteins were removed from the matrix.  
446 The 9 WT samples were then pooled into one WT group and the data was exported for  
447 further analysis with R version 3.3.1 (2016-06-21) and the package limma (21,23) (version  
448 3.28.14). Limma was then used to calculate the log<sub>2</sub> fold change and the empirical Bayes  
449 p-Value between the following groups: AD\_9m & WT, AD\_5m & WT, AD\_3m & WT,  
450 WT\_9m & WT, WT\_5m & WT, and WT\_3m & WT.

## 451 **Acknowledgements**

452

## 453 **References**

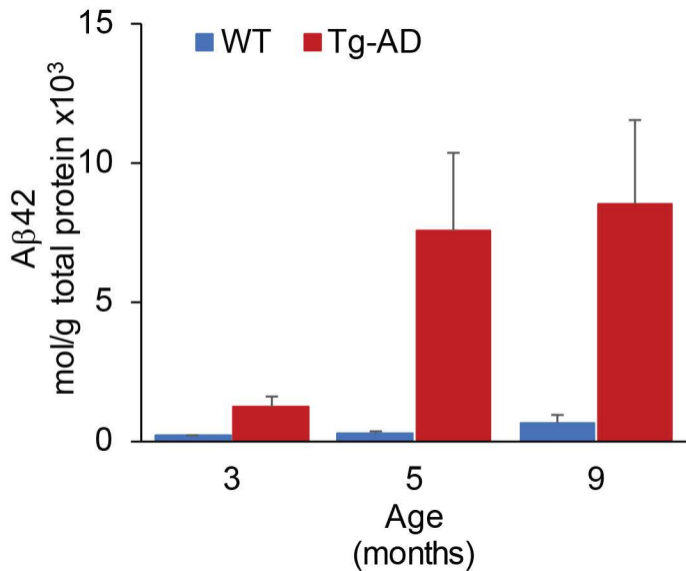
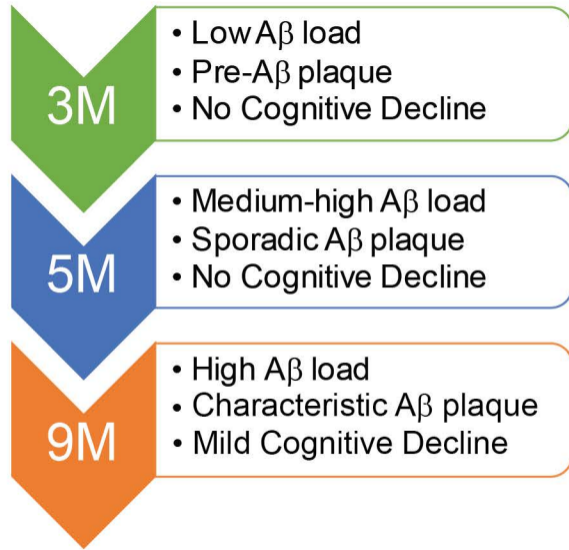
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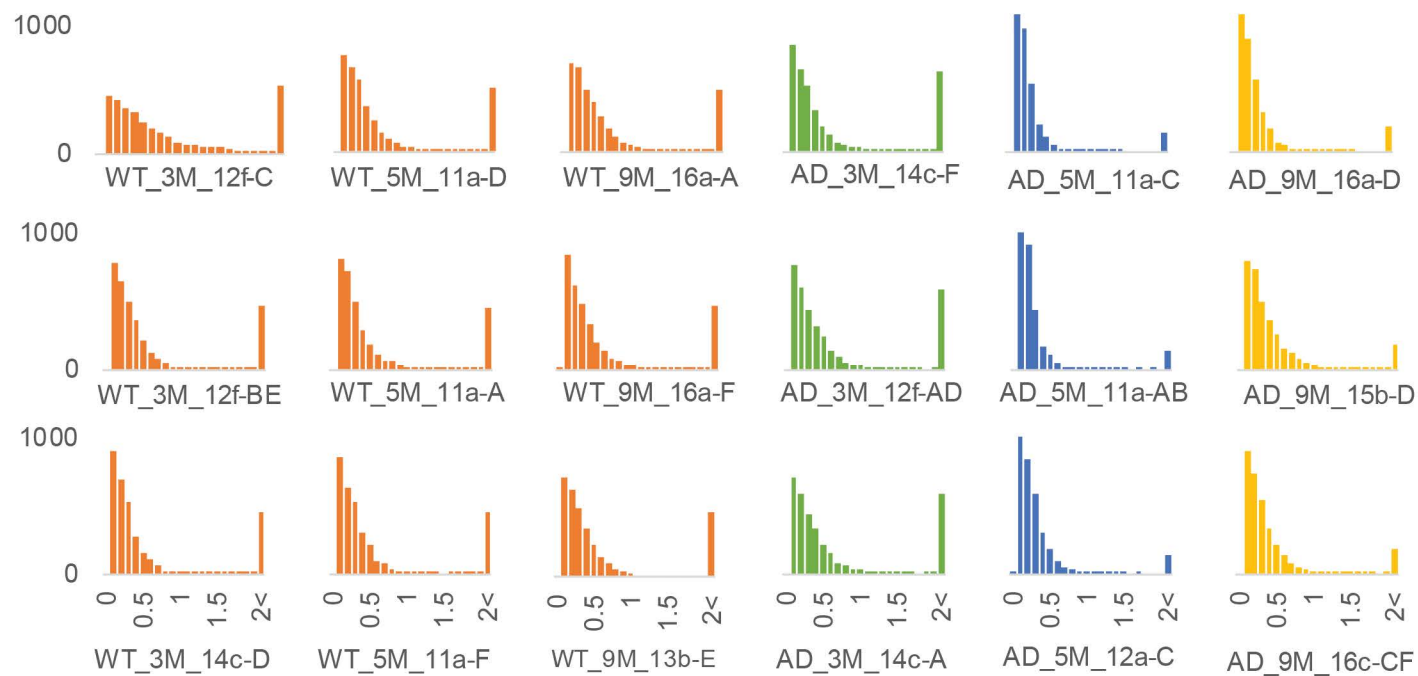
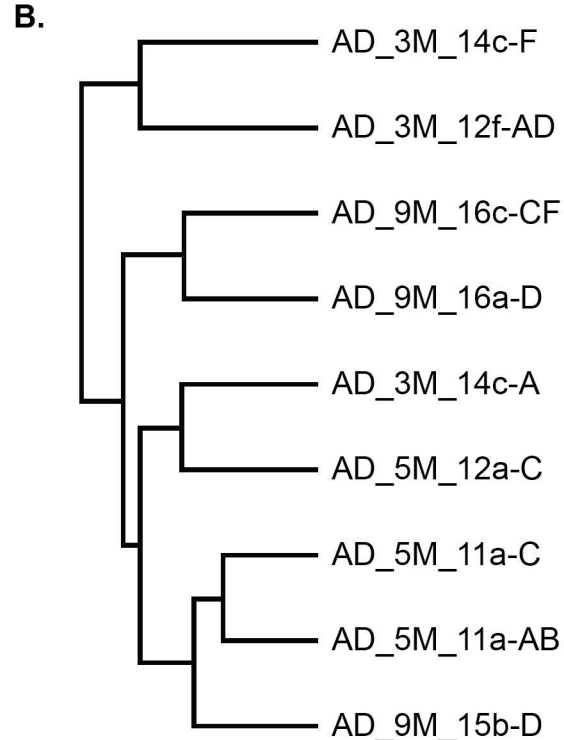
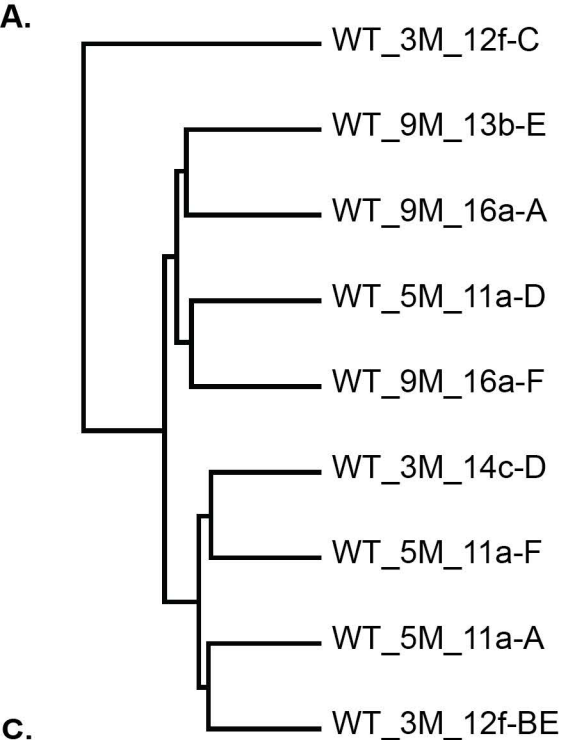
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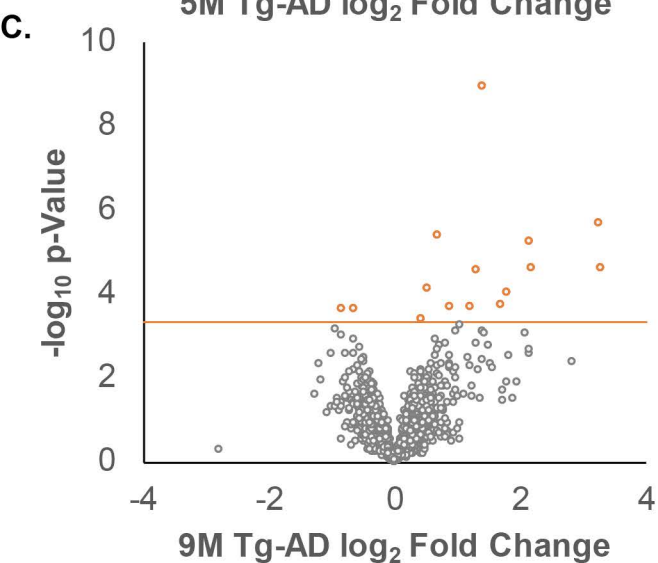
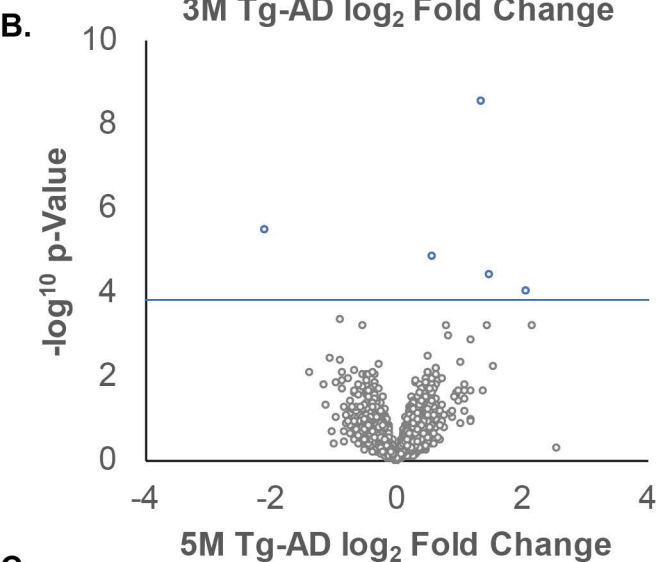
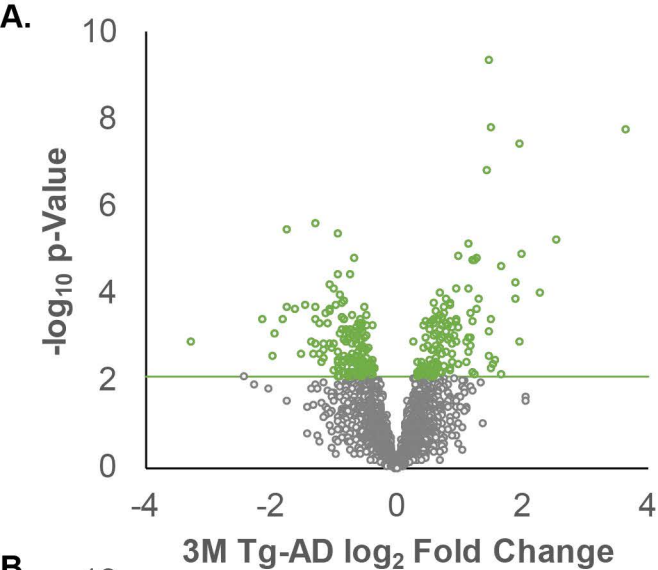
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- 561



**A.****B.**





AD Progression



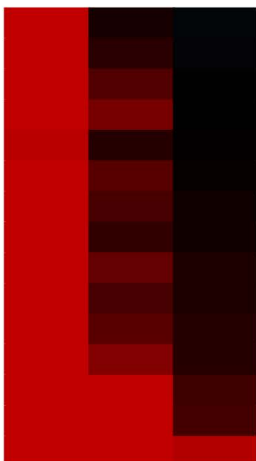
3M 5M 9M

\*APP  
ANKLE2  
AGO1

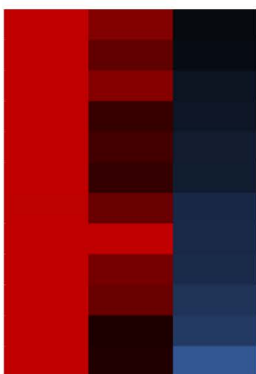


1: Up regulated

TMEM35  
RPS8  
CA4  
ABCF2  
TMEM214  
PRMT5  
PPM1E  
\*NDUFV3  
LYPLAL1  
\*RTN4RL1  
TALDO1  
\*SYVN1  
SH3BP1  
RELL2  
TMEM240

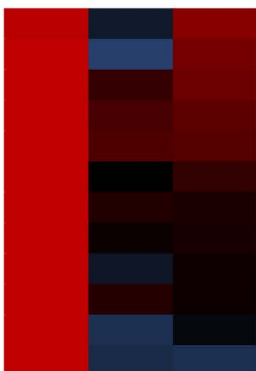
2: Up regulated  
Decreasing

\*NDUFA7  
\*TMCC2  
DNAJB14  
GAK  
GPC4  
ZFYVE1  
ARFIP2  
SYT5  
OSBPL11  
PIGT  
RPL37A  
ZDHHC17



3: Decreasing

GRID2  
SCARB2  
\*SHC3  
NUDT3  
CRKL  
TMEM132E  
GABARAPL1  
TXNRD2  
NAA35  
RPS11  
PIGK  
TTC19



4: Up in 3M

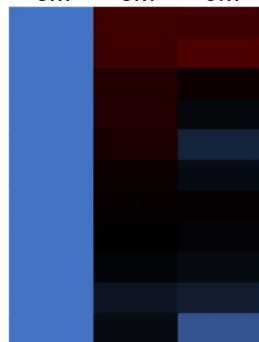
log<sub>2</sub> Fold Change Color Key

AD Progression



3M 5M 9M

GABRA3  
PLS1  
GLRA1  
MRPS30  
\*IDE  
MPC1  
NECAP1  
TUBA8  
CCSMST1  
DCTN3  
ABCD2



5: Down in 3M

GCSH



6: Down in 5M

VTN

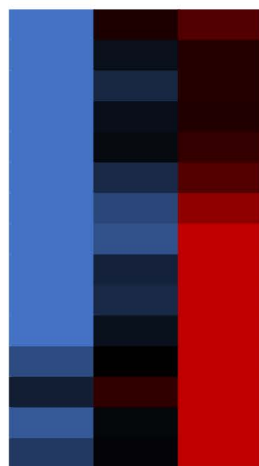


7: Up in 9M

\*APOE

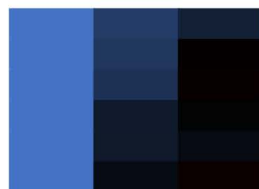


CACNG7  
DAAM2  
GJB6  
TPD52  
NSMF  
EEF1D  
VIM  
LMNA  
LMNB1  
\*UQCRH  
H3F3A  
H2AFV  
HIST2H3A  
TMPO  
HNRNPA2B1



8: Increasing

ABHD3  
GRIK2  
SUGT1  
CECR5  
VCAM1  
OMG

9: Down regulated  
Increasing

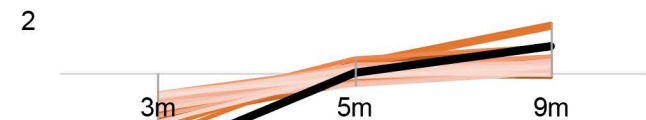
CDK18  
GRM8  
\*NDUFA4  
VPS16  
CNNM2  
TRAPPC9  
FRRS1L



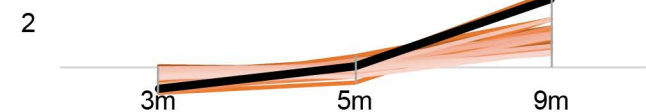
10: Down regulated

**A.**

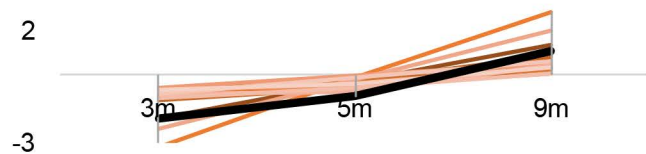
$(-3, 0, 1)$   
P-Value =  $3E-15$

**B.**

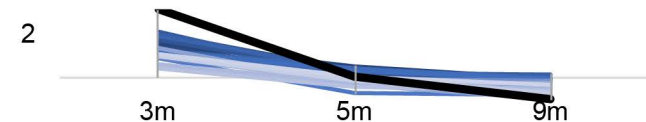
$(-1, 0, 3)$   
P-Value =  $2E-06$

**C.**

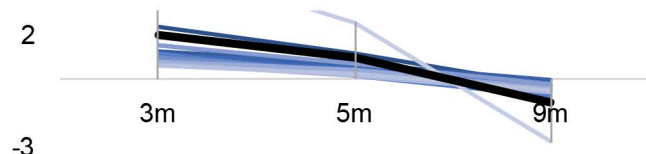
$(-2, -1, 1)$   
P-Value =  $2E-05$

**D.**

$(3, 0, -1)$   
P-Value =  $3E-05$

**E.**

$(2, 1, -1)$   
P-Value =  $3E-04$



## Canonical Pathway

EIF2 Signaling

Phospholipase C Signaling

RhoA Signaling

Actin Cytoskeleton Signaling

Endothelin-1 Signaling

Signaling by Rho Family  
GTPases

PPAR $\alpha$ /RXR $\alpha$  Activation

Glutamate Receptor Signaling

Mitochondrial Dysfunction

Oxidative Phosphorylation

## p-Value

3m

(FDR=0.1  
FC=1)

3m

(FDR = 0.1)

3m

(FDR = 0.1,  
FC  $\geq$  0.4,  
+STEM)

1.5

0.5

1.3

0.0

1.9

2.3

0.0

2.3

2.3

0.3

2.6

2.4

0.9

1.1

1.3

0.3

2.9

2.6

0.0

0.4

2.3

1.9

4.1

3.7

4.8

6.6

7.6

3.4

5.0

5.5

0

2

4

6

8

10

p-Value Color Key

## z-Score

3m

(FDR=0.1  
FC=1)

3m

(FDR = 0.1)

3m

(FDR = 0.1,  
FC  $\geq$  0.4,  
+STEM)

N/A

N/A

2.2

0.0

1.0

1.9

0.0

1.6

1.9

N/A

1.0

1.2

N/A

0.4

0.7

N/A

0.7

0.6

0.0

N/A

-1.7

N/A

-0.4

-0.4

N/A

N/A

N/A

N/A

N/A

N/A

-2.0

-1.0

-0.5

0.0

0.5

1.0

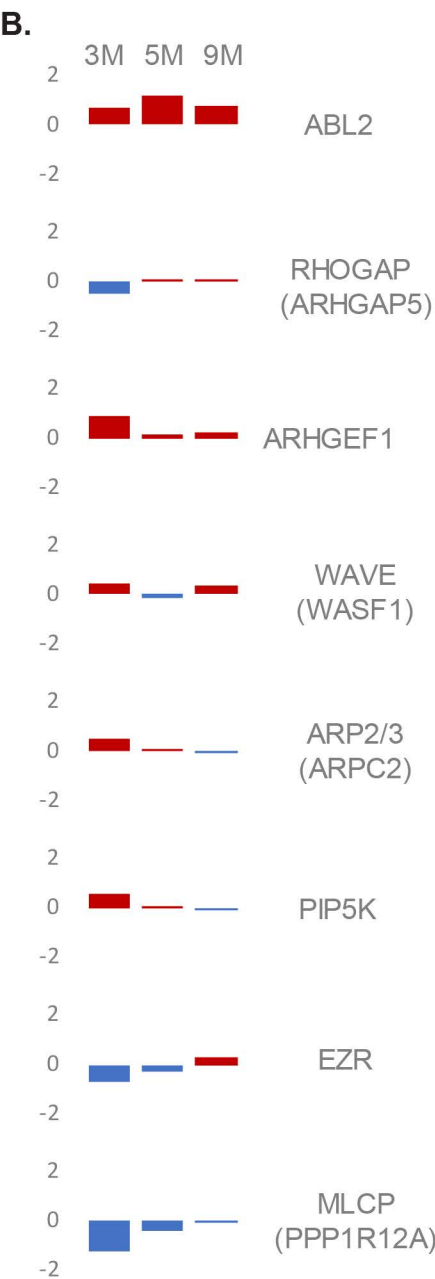
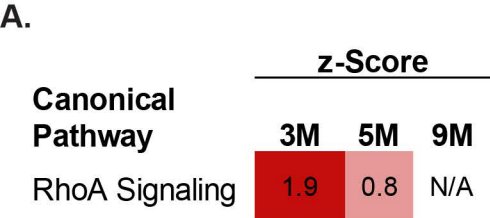
2.0

z-Score Color Key

Canonical Pathway	STEM enriched		
	3m	5m	9m
RhoA Signaling	1.9	0.8	N/A
Phospholipase C Signaling	1.9	2.4	1.3
Actin Cytoskeleton Signaling	1.2	0.3	1.7
EIF2 Signaling	2.2	1.3	-1.3
$\alpha$ -Adrenergic Signaling	0.8	1.6	-0.4
Thrombin Signaling	0.4	1.1	-0.4
Signaling by Rho Family GTPases	0.6	-0.3	1.6
CREB Signaling in Neurons	0.7	-0.7	1.1
NRF2-mediated Oxidative Stress Response	1.3	-0.4	0.4
IL-1 Signaling	1.0	-1.0	N/A
Synaptic Long Term Potentiation	0.8	-0.4	-0.4
Synaptic Long Term Depression	0.4	-1.1	-1.3
G Beta Gamma Signaling	1.3	-0.4	-1.0
Huntington's Disease Signaling	1.0	0.0	-1.0
Endothelin-1 Signaling	0.7	0.0	-1.3
fMLP Signaling in Neutrophils	1.0	N/A	N/A
Ephrin Receptor Signaling	0.8	0.0	0.0
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.4	N/A	N/A
Integrin Signaling	-0.3	0.8	1.1
Calcium Signaling	0.0	0.4	1.6
Insulin Receptor Signaling	-0.4	1.1	-0.4
RhoGDI Signaling	-0.7	0.4	N/A
PPAR $\alpha$ /RXR $\alpha$ Activation	-1.7	-1.0	2.1
Glutamate Receptor Signaling	-0.4	-1.3	1.0
Apoptosis Signaling	-1.0	-1.0	1.0
LPS/IL-1 Mediated Inhibition of RXR Function	0.0	N/A	-1.0
Gai Signaling	-1.3	-0.4	-2.0

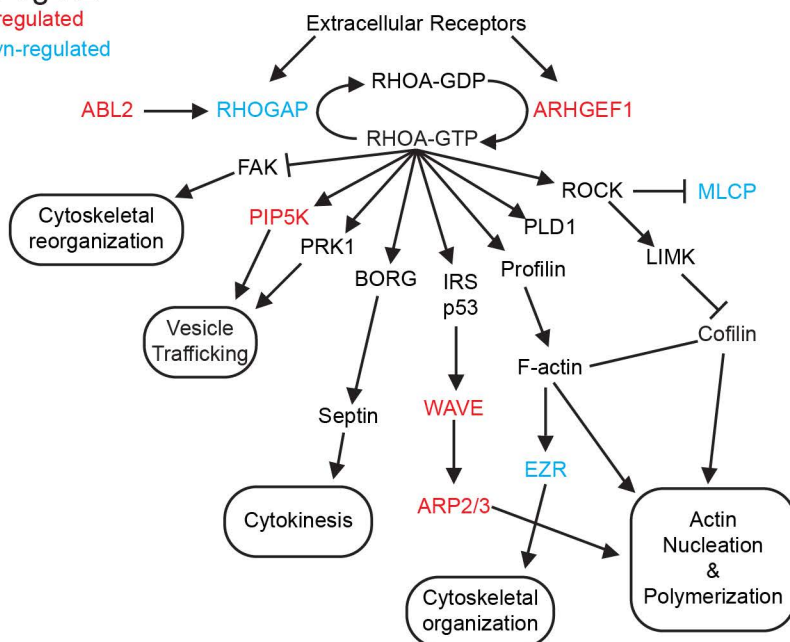
  

-2.0	-1.0	-0.5	0.0	0.5	1.0	2.0
z-Score Color Key						



**C. 3m Tg-AD**

Up-regulated  
Down-regulated

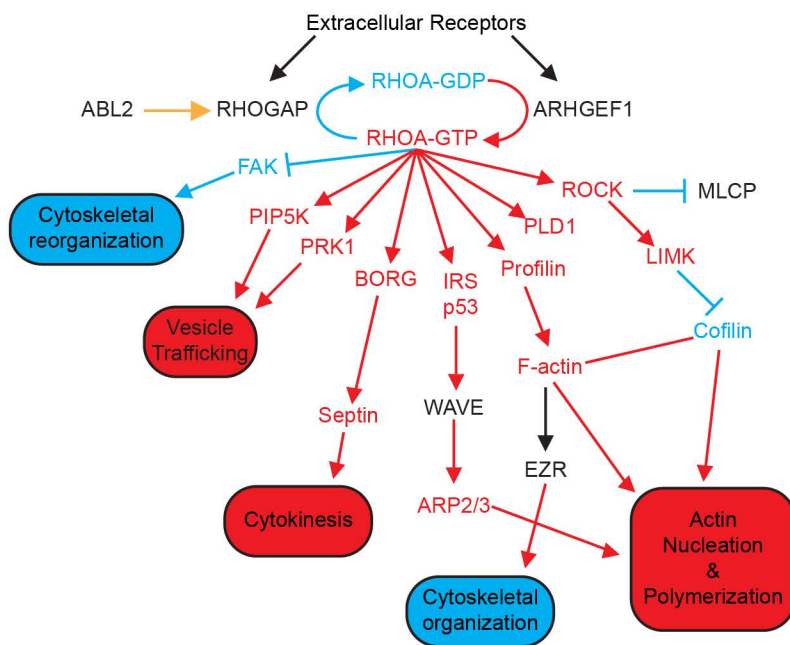


**D. 3m Tg-AD IPA Molecule Activation Predictor**

Activated

Inhibited

Inconsistent with state of downstream molecule





# A $\beta$ impact on Cofilin & Actin Stabilization

Activated

Inhibited

Inconsistent with state of downstream molecule

