

1 **PLD3 gene and processing of APP**

2

3 Authors:

4 Pietro Fazzari^{1,2,+}, Katrien Horre^{1,2}, Amaia M. Arranz^{1,2}, Carlo Sala Frigerio^{1,2},5 Takashi Saito^{3,4}, Takaomi C Saido³, Bart De Strooper^{1,2,5*}

6 Publication type: Brief Communication Arising

7 ARISING FROM, C. Cruchaga et al. Nature 505, 550–554 (2014); doi:10.1038/nature12825

8 (1) VIB Center for the Biology of Disease, Leuven, Belgium

9 (2) Center for Human Genetics, Leuven Institute for Neurodegenerative
10 Disorders (LIND) University Hospitals Leuven, and University of Leuven,
11 O&N4 Herestraat, Leuven, Belgium12 (3) Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Wako-shi, Saitama,
13 Japan

14 (4) Japan Science and Technology Agency, Saitama, Japan

15 (5) UCL Institute of Neurology, Queen Square, London, UK

16

17 Pietro Fazzari, Pietro.Fazzari@med.kuleuven.be18 Katrien Horre, Katrien.Horre@cme.vib-kuleuven.be19 Amaia M. Arranz, amaia.arranz@cme.vib-kuleuven.be20 Carlo Sala Frigerio, Carlo.SalaFrigerio@cme.vib-kuleuven.be21 Takashi Saito, takasai@brain.riken.jp22 Takaomi C Saido, saido@brain.riken.jp23 ⁺ Current affiliation: CBM Severo Ochoa Department of Molecular Neurobiology, CSIC / UAM,
24 Madrid, Spain. Mail: pfazzari@cbm.csic.es

25

26 * Correspondence should be addressed to Professor Bart De Strooper:

27

28 bart.destrooper@cme.vib-kuleuven.be

29

30 VIB Center for the Biology of Disease

31 KU Leuven,

32 O&N 4, 6e verd

33 Campus Gasthuisberg

34 Herestraat 49, bus 602

35 3000 LEUVEN, Belgium

36

37 Phone: +32 16 37 32 46

38

39 Cruchaga *et al.*¹ recently showed that i) variants in the phospholipase D3 (*PLD3*) gene
40 confer increased risk for the development of Alzheimer's disease (AD), ii) *PLD3* expression
41 is decreased in sporadic AD patients and iii) the expression of *PLD3* inversely correlates
42 with the expression of Amyloid Precursor Protein (APP) and the production of A β peptides *in*
43 *vitro* in cell lines. Altogether, the genetic and functional data led the authors to conclude that
44 *PLD3* loss-of-function confers an increased AD risk by affecting APP processing. Here we
45 tested the relevance of *Pld3* for APP processing *in vivo* in physiological conditions and in an
46 AD-relevant model: *Pld3* deficiency did not affect App metabolism or amyloid plaque burden.
47 *PLD3* is however localized in the lysosomal compartment and its loss-of-function alters its
48 morphology, suggesting alternative functions for *PLD3* in neurodegeneration.

49 To test the effect of loss-of-function of *PLD3* on brain physiology we acquired the
50 *Pld3*^{tm1e(EUCOMM)Wtsi} mice (*Pld3*^{ko})². These mice carry a trapping cassette with a splicing
51 acceptor followed by a IRES:lacZ knocked-in in the *Pld3* gene (Extended Data Fig. 1a). This
52 insertion abrogates the expression of *Pld3* and results in the expression of the β -
53 galactosidase reporter instead (Extended Data Fig. 1b). Western blot (WB) analysis (not
54 shown) and X-gal staining showed that *Pld3* is highly expressed in the pyramidal neurons of
55 cortex and hippocampus; conversely, expression of the β -galactosidase was not detectable
56 in the interneurons of the hippocampus nor in the glial cells of the corpus callosum
57 (Extended Data Fig. 1d, 1d', 1d'' and 1d'''). Morphometric analysis of the gross morphology
58 of *Pld3*-deficient brains did not reveal any major abnormalities as compared to controls
59 (Extended Data Fig. 1e and 1f).

60 We investigated the effect of genetic deletion of *Pld3* on App proteolysis. mRNA levels of
61 *App* and different candidate genes, including *Adam10*, *Bace1* and the γ -secretase complex
62 subunits, are unchanged in *Pld3*-deficient cortices relative to control brains (Extended Data
63 Fig. 1c). Moreover, WB analysis shows that the expression of App full length (App FL), App
64 C-terminal fragments (App CTF) and the ratio between App CTF and App FL are not
65 affected by *Pld3* deletion in neither the cortex nor the hippocampus (Fig. 1a, b and Extended
66 Data Fig 1g,h). Finally, we assessed the levels of A β 40 and A β 42 in control and *Pld3*-
67 deficient mice by performing enzyme-linked immunosorbent assays (ELISA). Significantly,
68 genetic deletion of *Pld3* does not affect endogenous A β 40 and A β 42 generation in the cortex
69 and hippocampus of 3-month old adult mice (Fig. 1b and Extended Data Fig. 1h).

70 To establish the relevance of *Pld3* in an AD-relevant model, we crossed *Pld3*^{ko} mice with
71 App knock-in mice (*App*^{ki}) which have a humanized A β sequence in the endogenous App
72 gene and contains three clinical relevant mutations to increase amyloidogenesis³. The model
73 proposed by Cruchaga *et al.* predicts that loss of *Pld3* function would increase amyloid
74 burden. Consistent with the experiments above, *Pld3* deletion did not alter the levels of App
75 FL, App CTFs nor the App CTF/App FL ratio (Fig. 1c,d). The levels of A β 40 and A β 42 in tris-
76 buffered saline (TBS)-soluble and GuanidineHCl (Gu)-soluble fractions were not increased
77 in *Pld3*^{ko};*App*^{ki} compared to control mice (Fig. 1d and Extended Data Fig. 1i). Most
78 importantly, *Pld3* deletion did not have any effect on amyloid plaques burden (Fig. 2a,b)
79 further confirming that *Pld3* does not affect App metabolism in this AD-relevant mouse
80 model. Future studies will be required to investigate the effect of *Pld3* deletion in aged mice
81 or in other AD-models (e.g. tau or ApoE models).

82 We tried then to replicate the *in vitro* studies of Cruchaga *et al.* using transiently
83 expressing *PLD3* wild-type (WT), the catalytically inactive *PLD3* K418R (KR)⁴ and the AD-

84 linked PLD3 V232M (VM) variant in HEK293T cells stably expressing human APP-WT. The
85 results of these experiments turned out to be contradictory and highly variable. For instance
86 and consistently with the work of Cruchaga *et al.*, we observed a reduction in A β levels in
87 cells expressing the PLD3 WT and mutant proteins (Extended Data Fig. 2a). In contrast to
88 Cruchaga *et al.*, APP FL were however not significantly affected by PLD3 expression
89 (Extended Data Fig. 2b,c). Unexpectedly, the expression of AD-associated PLD3 VM
90 variant, proposed by Cruchaga *et al.* as loss-of-function allele, also decreased A β
91 generation. Notably, the overexpression of PLD3 in these experiments was >50 fold higher
92 than the endogenous expression (not shown). We reasoned that the discrepancies in the
93 results are likely explained by overexpression artefacts e.g. huge overexpression may lead
94 to mislocalization of the protein-of-interest resulting in non-physiological interactions.
95 Therefore, we further investigated the functional relevance of changes in PLD3 expression
96 by utilizing milder overexpression conditions to more closely mimic a physiologically relevant
97 context. We expressed decreasing amounts of PLD3 WT and the KR and VM mutants by
98 Neon[®] electroporation in HEK293T cells to allow for a more uniform expression of the PLD3
99 protein (about 20, 5 and 2 fold the endogenous level, Fig. 1u short and long exposures for
100 PLD3). In this experimental paradigm, the expression of PLD3 WT and mutants does not
101 reduce neither A β levels (Fig.1t) nor APP expression (Fig. 1u and 1f) confirming that the
102 reported effects of Pld3 on APP reported in Cruchaga *et al.* are artefacts due to huge
103 overexpression of PLD3.

104 Several AD-linked genes, that do not directly control APP processing, converge on the
105 regulation of endosomal-autophagic-lysosomal function⁵, although the etiological role of
106 endosomal-lysosomal impairment in AD is not completely understood^{6,7}. Notably, defects in
107 the endosomal-lysosomal system were found in AD brains⁸. Thus, we investigated the
108 localization of Pld3 in early endosomes (stained with Early Endosome Antigen 1 (EEA1)
109 marker) and in late endosomes and lysosomes (visualized with Lysosomal-associated
110 membrane protein 1 (LAMP-1) marker). Pld3 was not enriched in early endosomes but was
111 mostly localized to LAMP1 positive compartments (Extended Data Fig. 3a-d). Hence, we
112 analysed by electron microscopy (EM) the ultrastructure of lysosomes in *Pld3*^{ko} neurons.
113 Primary and secondary lysosomes of *Pld3* deficient CA1 neurons displayed an increase in
114 density, size and in total area occupied (Extended data Fig. 3e-g). Moreover, several of
115 these secondary lysosomes showed electron-transparent inclusions compatible with lipid
116 droplets (16 out of 73 secondary lysosomes in *Pld3*^{ko} vs 1 out of 22 in control brains). The
117 identification of the precise mechanisms underpinning these alterations is out of the scope of
118 the current study, nonetheless these results show that Pld3 is required to preserve the
119 structure of lysosomes *in vivo*.

120 In sum, our *in vivo* studies demonstrate that Pld3 is not relevant for App metabolism
121 neither in wild type mice nor in a model of AD pathology. These data challenge the
122 mechanistic model proposed by Cruchaga *et al.* and suggest a more complex role of PLD3
123 in the etiology of AD. Our findings that PLD3 protein is localized in and affects the
124 morphology of the lysosomal system indicate that PLD3 may be involved in the
125 pathophysiology of AD by exacerbating the known impairments of endosomal-lysosomal
126 systems⁵.

127

128

129
130

References

- 131 1. Cruchaga, C. *et al.* Rare coding variants in the phospholipase D3 gene confer risk for
132 Alzheimer's disease. *Nature* **505**, 550–4 (2014).
133
- 134 2. Skarnes, W. C. *et al.* A conditional knockout resource for the genome-wide study of
135 mouse gene function. *Nature* **474**, 337–342 (2011).
136
- 137 3. Saito, T. *et al.* Single App knock-in mouse models of Alzheimer's disease. *Nat*
138 *Neurosci* **17**, 661–663 (2014).
139
- 140 4. Osisami, M., Ali, W. & Frohman, M. a. A role for phospholipase D3 in myotube
141 formation. *PLoS One* **7**, (2012).
142
- 143 5. Nixon, R. a. The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–
144 997 (2013).
145
- 146 6. Boland, B. *et al.* Macroautophagy is not directly involved in the metabolism of amyloid
147 precursor protein. *J. Biol. Chem.* **285**, 37415–37426 (2010).
148
- 149 7. Lee, S., Sato, Y. & Nixon, R. A. Lysosomal Proteolysis Inhibition Selectively Disrupts
150 Axonal Transport of Degradative Organelles and Causes an Alzheimer's-Like Axonal
151 Dystrophy. *J. Neurosci.* **31**, 7817–7830 (2011).
152
- 153 8. Cataldo, a M., Hamilton, D. J., Barnett, J. L., Paskevich, P. a & Nixon, R. a.
154 Properties of the endosomal-lysosomal system in the human central nervous system:
155 disturbances mark most neurons in populations at risk to degenerate in Alzheimer's
156 disease. *J. Neurosci.* **16**, 186–199 (1996).
157
- 158 9. Morishima-Kawashima, M. *et al.* Effect of apolipoprotein E allele epsilon4 on the initial
159 phase of amyloid beta-protein accumulation in the human brain. *Am. J. Pathol.* **157**,
160 2093–9 (2000).
161

162 Authors contribution

163 PF, conceived the project, planned the experiments, performed the experiments,
164 analysed the results and wrote the manuscript; KH, performed *in vitro* experiments,
165 and performed WB and ELISA analysis *in vitro* and *in vivo* (Fig. 1g-n and Fig. 1q-v);
166 AMA, performed EM experiments and helped to analyse the data; CSF, performed
167 and analysed qPCR experiments (Fig. 1b-c); TS, TCS, previously characterized and
168 provided App knock-in mice; BDS, conceived and supervised the project, wrote the
169 manuscript. All the authors revised the manuscript and helped with comments and
170 feedback.

171 Acknowledgements.

172 cDNAs for human PLD3 WT and KR were kindly provided by Prof. Frohman. This work

173 was supported by the European Research Council (ERC) ERC-2010-AG_268675 to BDS,
174 the Fonds voor Wetenschappelijk Onderzoek (FWO), the KU Leuven and VIB, a
175 Methusalem grant of the KU Leuven/Flemish Government to BDS. BDS is supported by the
176 Bax-Vanluffelen Chair for Alzheimer's Disease and "Opening the Future" of the Leuven
177 Universiteit Fonds (LUF). Confocal microscope equipment was acquired through a Hercules
178 Type 1 AKUL/09/037 to W. Annaert. Mouse experiments were supported by Inframouse
179 (KU Leuven and VIB infrastructure).

180 **Material and methods**

181
182 HEK293T cells were transfected with TransIT-LT1® (Mirusbio, USA, product nos.MIR2300)
183 or electroporated with Neon® (Invitrogen, Catalog Number MPK5000) according to
184 manufacturer instructions. *Pld3*^{tm1e(EUCOMM)Wtsi} mice (*Pld3*^{ko}) were obtained from The
185 European Mouse Mutant Archive-EMMA. *App*^{NL-G-F/NL-G-F} knock-in mice (*App*^{ki}) that carry the
186 human A β sequence with triple Swedish, Arctic and Beyreuther/Iberian mutations were
187 generated as described³. Detailed experimental procedures are provided in Supplemental
188 Materials and Methods.

189 **Supplemental material and methods**

190 cDNA for PLD3 VM was generated by site directed mutagenesis and verified by
191 sequencing.

192 Cell lysates were prepared in STE with 1% Triton X-100. Densitometric quantification of
193 WB were normalized for Actin levels. For the ELISA we collected cell supernatant and
194 measured for A β by standard techniques. Data obtained were corrected for the levels of total
195 intracellular protein measured by BCA.

196 For quantitative PCR, RNA was retrotranscribed to cDNA with oligo dT primers using the
197 SUperscript II Reverse transcriptase kit (Invitrogen). Real-time PCR was carried out using
198 the LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler LC480 (Roche)
199 instrument.

200 For β -galactosidase activity brains were perfused with PBS, snap frozen and cut at
201 cryostat. 10 μ m sections were fixed for 10 minutes in 0.2 % glutaraldehyde, rinsed and
202 incubated in X-Gal staining solution according to standard procedure.

203 Samples from control and *Pld3*^{ko} mice were lysate in 0.4% Diethylamine, 50 mM NaCl, 50
204 mM Tris-HCl buffer with EDTA free protease inhibitors and processed for WB or ELISA. WB
205 densitometry and ELISA were normalized as for cells lysates above. Brains from control and
206 *Pld3*^{ko}; *App*^{ki} mice were perfused in PBS. Next, half brain was postfixed in 4% PFA, rinsed,
207 cut at 100 μ m at the vibratome and processed for thioflavin staining; the other half was
208 homogenised in tissue protein extraction reagent (Pierce) with protease inhibitors. Extraction
209 of TBS- and Gu-soluble A β was performed as described⁹.

210 Thioflavin staining was performed according to standard technique, for confocal imaging
211 we used Olympus FV1000 IX2 Inverted Confocal microscope with 20x UPlanSapo. Images
212 were automatically thresholded and quantified with ImageJ.

213 Colabelling of PLD3 in HEK293 cells with EEA1 and LAMP1 was done with the antibodies
214 anti-PLD3 (Sigma, #HPA012800; dilution 1/200), anti-EEA1 (BD, #610456; 1/500) and anti-
215 LAMP1 (Santa cruz, #sc-19992; 1/500) according to standard IF techniques. Samples were
216 fixed in PFA 4% for 10 minutes. Pictures were taken with an Olympus FV1000 IX2 Inverted
217 Confocal microscope with 60x UPlanSapo. Generation of PDM image and quantification of
218 Pearson's and Mander's coefficients was performed using the ImageJ Plug-in "Intensity
219 Correlation Analysis".

220 For EM analysis, *Pld3*^{ko} mice and control littermates were perfused at 1 month with 2.5%
221 glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer. 300 μ m coronal brain
222 sections were cut on a vibratome and rectangular pieces of tissue comprising the CA1
223 region were dissected. Briefly, the tissue was post-fixed with 1% OsO₄, 1.5% K₄Fe(CN)₆ in
224 0.1 M cacodylate buffer, rinsed, stained with 3% uranyl acetate and dehydrated in graded

225 ethanols and propyleneoxide, followed by embedding in EMBED812. 70 nm ultrathin sections
226 were mounted on copper grids and imaged at 3000x using a JEM-1400 transmission
227 electron microscope (Jeol).
228
229

230 **Figure legends**

231 **Figure 1**

232 **a**, WB of lysates from control and *Pld3^{ko}* show APP FL, APP CTF and Pld3 expression in
233 cortex at 3 months.

234 **b**, Densitometry of WB and ELISA quantification A β 40 and A β 42 levels in cortex from
235 *Pld3^{ko}* mice relative to control mice. n>7 control and n=10 *Pld3^{ko}* mice. Graphs show mean \pm
236 SEM.

237 **c**, WB of lysates from control and *Pld3^{ko};App^{ki}* show APP FL, APP CTF and *Pld3*
238 expression in cortex at 4 months.

239 **d**, Densitometry of WB and ELISA of TBS- A β 40 and A β 42. Graphs show mean \pm SEM.
240 n=8 for both Ctrl and *Pld3^{ko};App^{ki}*.

241 **Figure 2**

242 **a**, Confocal images of thioflavin stained cortices from control and *Pld3^{ko};App^{ki}* mice. Scale
243 bar 50 μ m.

244 **b**, Quantification of amyloid plaques burden as percentage of area occupied by the
245 plaques. Graphs show mean \pm SEM. n=8 for both Ctrl and *Pld3^{ko};App^{ki}*.

246

247 Extended Data Figure legends

248 Extended Data 1

249 **a**, Schema of the *Pld3* targeted inactivation and LacZ-tagging strategy in *Pld3^{ko}* mice. The
250 trapping cassette with a splicing acceptor followed by a IRES:lacZ knocked-in was inserted
251 between exon 9 and 10. Coloured bars indicate the primer binding sites of qPCRs (shown in
252 **b**). SA, splicing acceptor; IRES, internal ribosome entry site; neo, neomycine cassette.

253 **b**, Validation of *Pld3* inactivation by qPCR. The expression of exons 7-8 is strongly
254 reduced, while exons 9-10 are not detectable (nd) in *Pld3^{ko}* mice which express LacZ
255 instead at 1 month (e9-LacZ). Expression levels are plotted relative to respective reference.
256 nd, expression value < 0.005. n=6 for Ctrl and *Pld3^{ko}* mice; Graphs represent the mean \pm
257 SD; ***P<0.001. T test.

258 **c**, qPCR expression analysis of mRNA of *App* and of genes involved in its processing in
259 *Pld3^{ko}* relative to control mice at 1 month. n=6 for WT and *Pld3^{ko}* mice; Graphs represent the
260 mean \pm SD.

261 **d**, X-gal staining of *Pld3^{het}* brain show the expression of *Pld3* in neurons at 1 month. Fast
262 red is used as counterstaining. Cx, cortex; cc, corpus callosum; Hip, hippocampus; so,
263 stratum oriens; sp, stratum pyramidalis; sr, stratum radiatum; DG, dentate gyrus. Scale bar
264 500 μ m. I', I'' and I''' show insets from I in sp, so and cc respectively. Scale bar 20 μ m. I', X-
265 gal staining in CA1 pyramidal layer of the hippocampus. I'', I''', β -galactosidase activity is
266 not detectable in interneurons of the hippocampus (full arrowheads in I'') nor in the glia cells
267 of the corpus callosum (empty arrowheads in I''').

268 **e**, Dorsal, lateral; and central view of representative brains from control and *Pld3^{ko}* mice
269 at 3 months. w, width; l, length; h, height; t, thickness.

270 **f**, Morphometric quantification of **e**. Graphs show mean \pm SD. n=2 Ctrl and n=3 *Pld3^{ko}*
271 Scale bar 2 mm.

272 **g**, Representative WB of lysates from control and *Pld3^{ko}* show APP FL, APP CTF and
273 *Pld3* expression in hippocampus at 3 months.

274 **h**, Densitometric analysis of WB and ELISA quantification A β 40 and A β 42 levels in
275 hippocampus from *Pld3^{ko}* mice relative to control mice. n>7 control and n=10 *Pld3^{ko}* mice
276 from three litters. Graphs show mean \pm SEM.

277 **i**, ELISA of GU-soluble A β 40 and A β 42. Graphs show mean \pm SEM. n=8 for both Ctrl and
278 *Pld3^{ko}*; *App^{ki}*, ** P<0.01, two-way ANOVA.

279 Extended Data 2

280 **a**, Enzyme-linked immunosorbent assay (ELISA) shows the levels of A β 40 and A β 42 in
281 the supernatant of HEK293T transfected with TransIT-LT1 to express either GFP as control
282 or PLD3 WT, PLD3 KR, and PLD3 VM. A β levels are expressed relative to control. Graph
283 shows mean \pm SEM. n=6 out of 2 experiments. ***, P<0.001. Two-way ANOVA.

284 **b**, Representative WBs show APP FL, APP CTF and PLD3 in lysates from HEK293T
285 cells from the experiment in **a**.

286 **c**, Densitometric analysis of WBs from the experiment in **q,r**. Graphs shows mean \pm SEM.
287 $n=6$ out of 2 experiments. *, $P<0.05$. Two-way ANOVA.

288 **d**, Levels of A β 40 and A β 42 in the supernatant of HEK293 cells electroporated with 2.5,
289 0.75, and 0.25 μ g of PLD3 WT, PLD3 KR, and PLD3 VM relative to GFP electroporated
290 control cells. Graphs shows mean \pm SEM. $n=6$ out of 3 experiments. *, $P<0.05$. Two-way
291 ANOVA.

292 **e**, Representative WBs show APP FL, APP CTF and PLD3 in lysates from **d**. For PLD3
293 short and long exposures of the same blot are shown.

294 **f**, Densitometry of WBs from **e**. Levels are relative to control. Graphs shows mean \pm SEM.
295 $n=6$ out of 3 experiments.

296 **Extended Data 3**

297 **a**, Confocal images show that PLD3 does not colocalize with EEA1 in early endosomes.
298 The inset shows area magnified in the right panels. Product of the difference of the mean
299 (PDM) image illustrates the negative correlation of PLD3 and EEA1 (light blue arrowheads).
300 The right bar shows colour codes for PDM values (negative in blue and positive in orange).
301 Scale bars 5 μ m.

302 **b**, Pearson coefficients for EEA1/PLD3 ($n=5$) and LAMP1/PLD3 ($n=7$). Graph shows
303 Means \pm SEM.

304 **c**, PLD3 is mostly localized in LAMP1 positive late endosomes/lysosomes. The inset
305 shows area magnified in the right panels. PDM image shows the positive correlation of PLD3
306 and LAMP1 staining (yellow arrowheads). Scale bars 5 μ m. Colour scales indicate PDM
307 values.

308 **d**, Mander's coefficients for LAMP1 and PLD3 co-labelling. Graph shows Means \pm SEM,
309 $n=7$.

310 **e**, Representative EM pictures of primary and secondary lysosomes (white and black
311 arrowheads respectively) from control and *Pld3*^{ko} deficient neurons. The arrow indicates
312 electron-transparent inclusion in a secondary lysosome. Scale bars: 500 nm.
313

314 **f**, Density, size and area occupied by primary lysosomes. Graph shows Means \pm SEM.
315 For density and area: Ctrl, $n = 50$ fields out of 2 brains; *Pld3*^{ko}, $n = 84$ out of 3 brains. For
316 density: * $p<0.05$, Mann Whitney test. For area: *** $p<0.001$, T test. For size: Ctrl: $n=91$;
317 *Pld3*^{ko}, $n=208$; *** $p<0.001$, T test.
318

319 **g**, Density, size and area occupied by secondary lysosomes. Graph shows Means \pm
320 SEM.

321 For density and area: Ctrl, $n = 50$ fields out of two brains; *Pld3*^{ko}, $n = 84$ out of 3 brains.
322 For density: * $p<0.05$, Mann Whitney test. For area: *** $p<0.001$, T test. For size: Ctrl: $n=22$;
323 *Pld3*^{ko}, $n=73$; * $p<0.05$, T test.

324

Figure 1

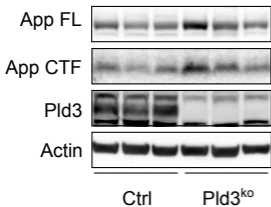
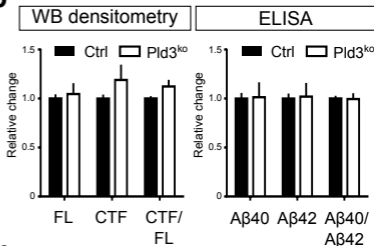
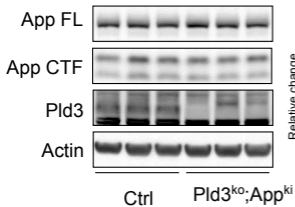
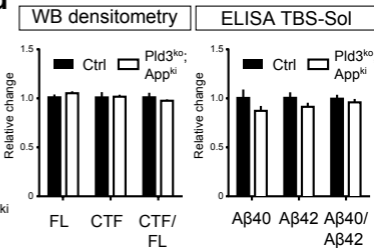
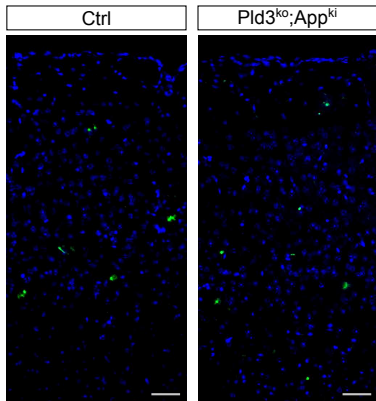
a**b****c****d**

Figure 2

a



b

