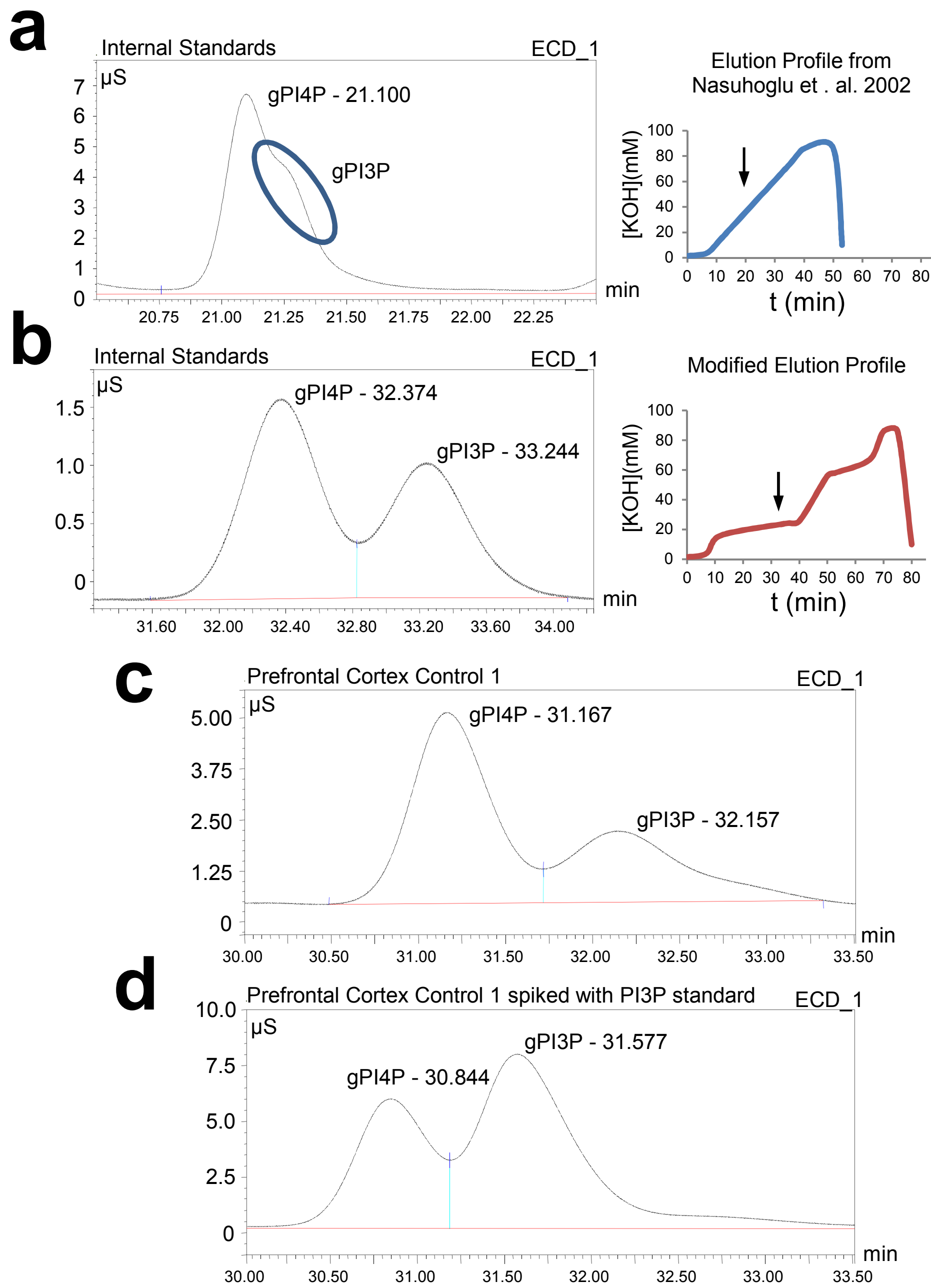
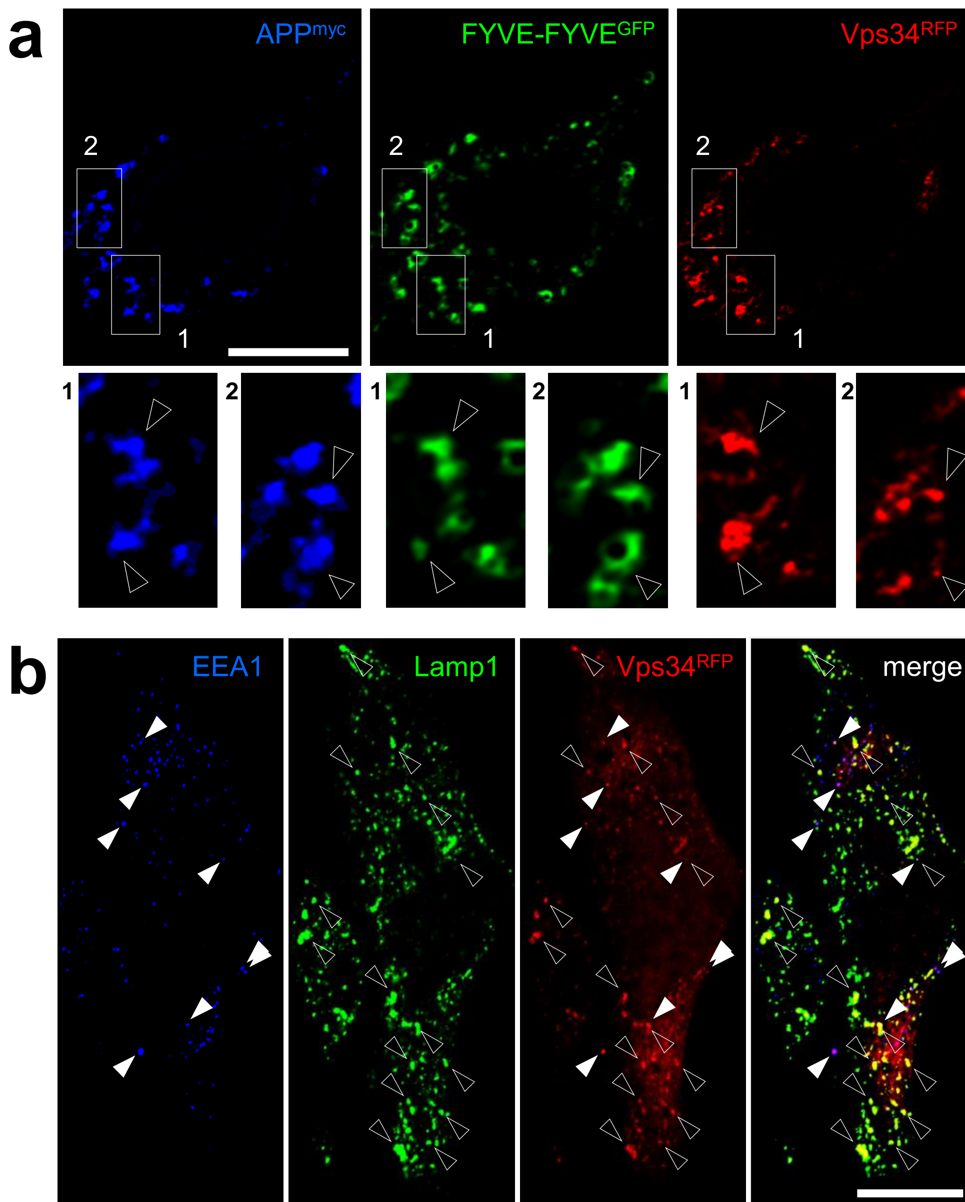


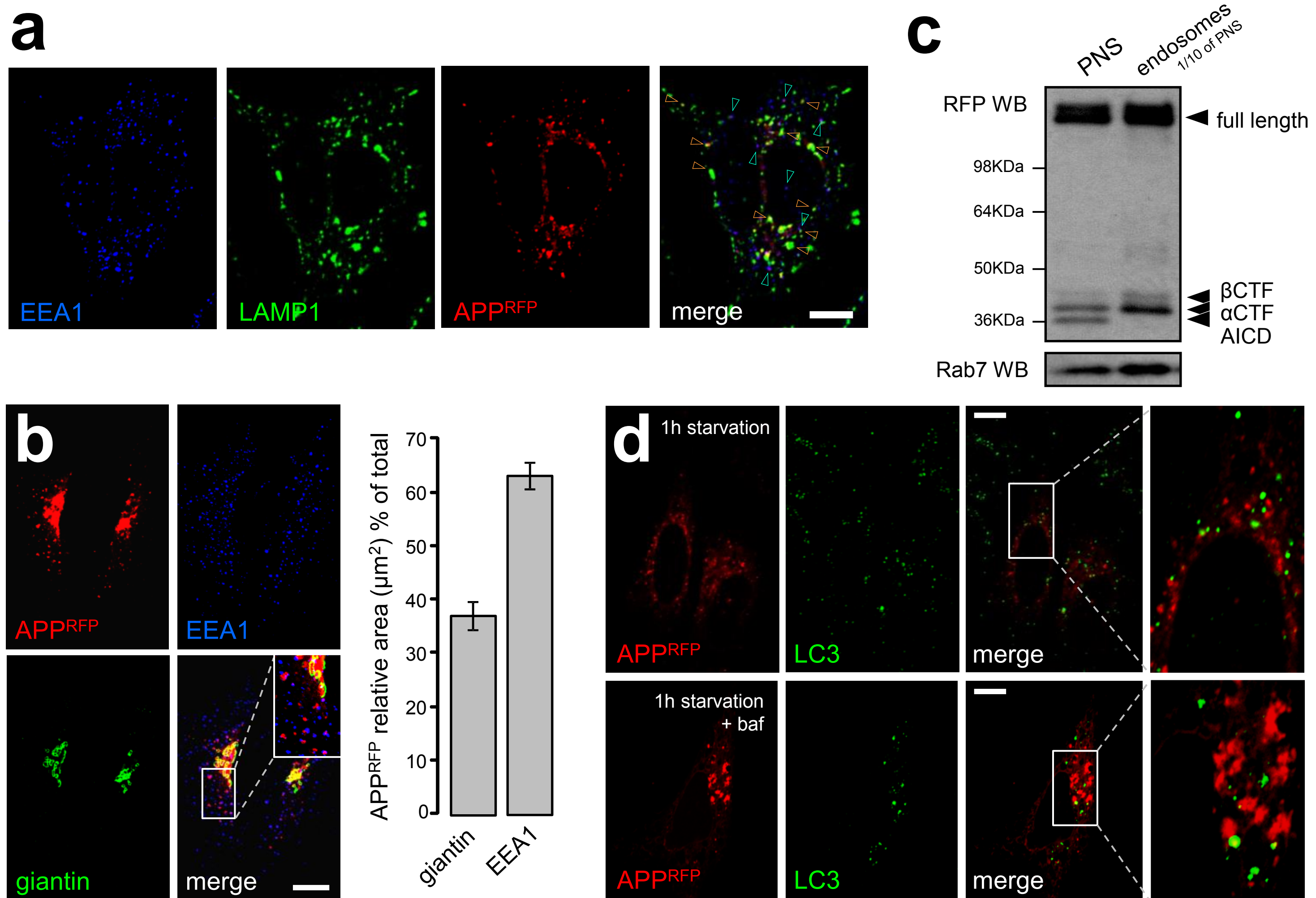
Supplementary Figure S1: Optimization of PI3P detection and analysis of control lipids in AD brains.

a to d) HPLC separation of deacylated PI4P and PI3P. **a)** The HPLC elution profile described in Nasuhoglu et. al. 2002 was unable to sufficiently separate PI3P from PI4P in a mixed internal standard preparation. The approximate elution time of PI4P and PI3P is indicated by the arrow. **b)** The HPLC elution profile was modified with a flatter elution gradient to enable separation of PI3P from PI4P. The approximate elution time of PI4P and PI3P is indicated by the arrow. **c)** Representative elution spectra of a prefrontal cortex control sample. **d)** The same prefrontal cortex sample spiked with PI3P internal standard.



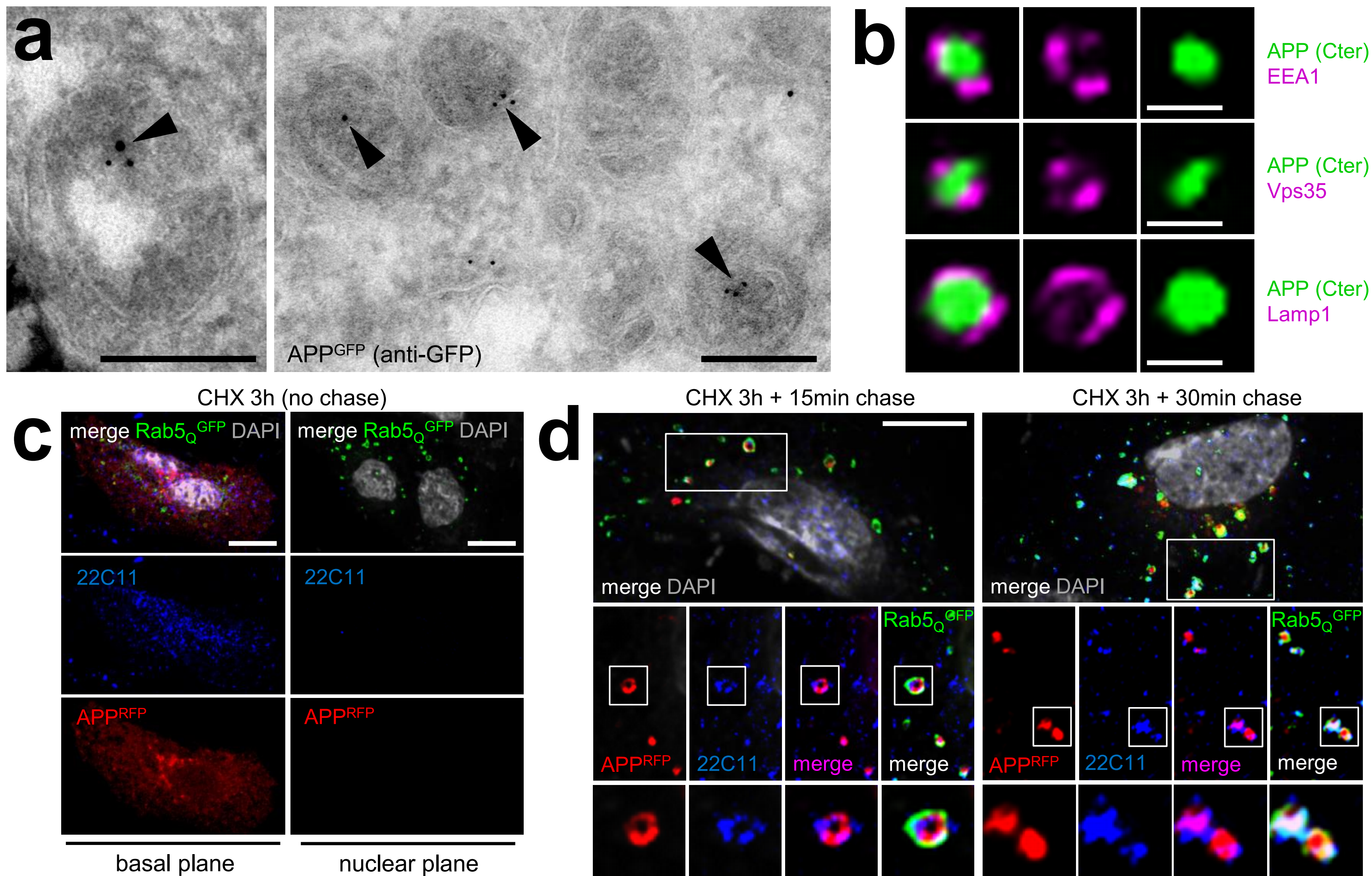
Supplementary Figure S2: APP is associated with Vps34- and PI3P-positive endosomes.

a) Confocal analysis of HeLa cells transfected with Vps34^{RFP}, APP^{myc} and FYVE-FYVE^{GFP}, fixed and labeled with anti-myc antibody. Insets 1 and 2 show magnifications where all three proteins are present on the same membranous structures (empty arrowheads). Scale bar = 10µm. **b)** Confocal analysis of HeLa cells transfected with Vps34^{RFP}, fixed and labeled with anti-EEA1 and anti-LAMP1 antibodies. Solid and empty arrowheads indicate colocalization in membranous structures between Vps34^{RFP} and the early endosomal marker EEA1 and the late endosomal marker Lamp1, respectively. Scale bar = 10µm.

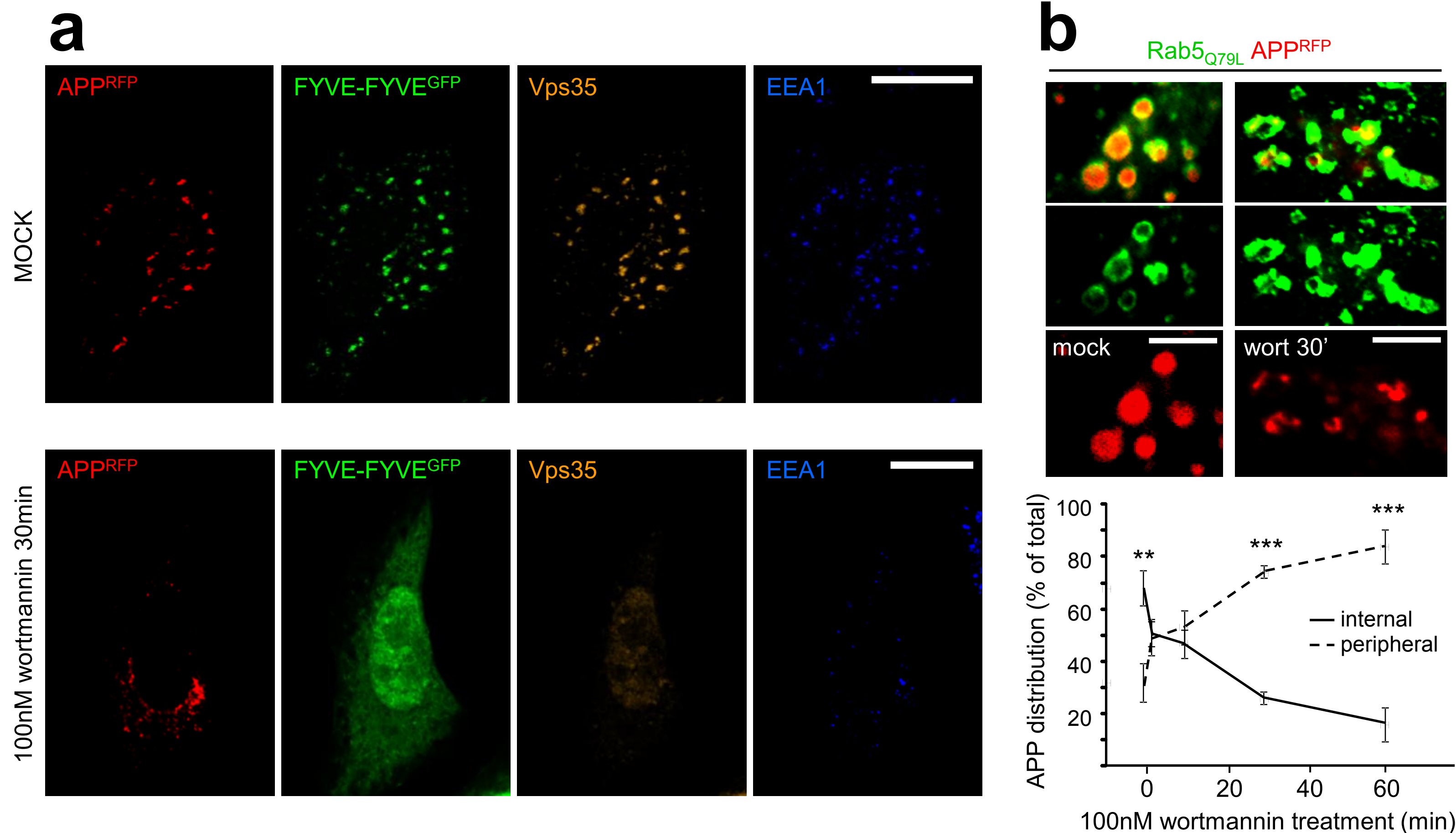


Supplementary Figure S3: Analysis of the subcellular localization of APP^{RFP}.

a) Confocal analysis of HeLa cells transfected with APP^{RFP} and labeled with the indicated antibodies. Colocalizations between APP^{RFP} and the early endosomal marker EEA1 are indicated (orange arrowheads) as well as those between APP^{RFP} and the late endosomal marker LAMP1 (blue arrowheads). **b)** Confocal analysis of HeLa cells transfected with APP^{RFP} and labeled with the indicated antibodies. APP^{RFP} colocalized with EEA1 and late Golgi marker giantin, as illustrated by quantification of relative RFP fluorescence colocalization with EEA1 or giantin (right panel, n = 30 cells). **c)** Total endosomal fraction was prepared from HeLa cells transfected with APP^{RFP} and analyzed by Western blot with anti-Rab7 and -RFP antibodies. Endosomal fraction represents 1/10 of the loaded post nuclear supernatant (PNS) in terms of total protein amount. Full length APP^{RFP} and its cleavage products [COOH-terminal Fragments (CTFs) and the APP intracellular domain (AICD) are shown. **d)** Confocal analysis of HeLa cells transfected with APP^{RFP} and starved for 1 h in the absence (upper panel) or presence (baf, lower panel) of 50 nM bafilomycin A1, before labeling with an anti-LC3 antibody. For a, b, d, scale bar = 10µm.

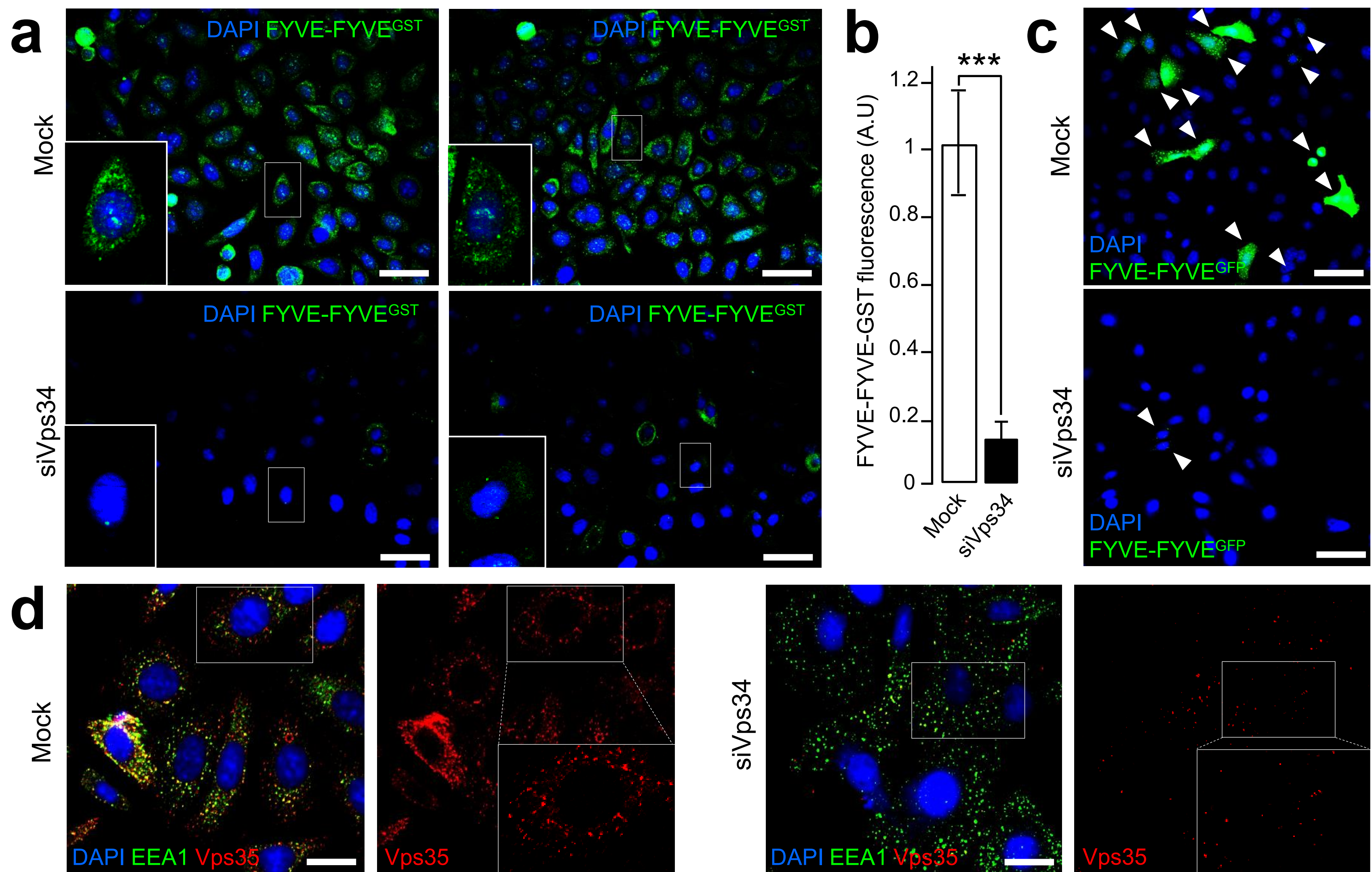


Supplementary Figure S4: Electron and light microscopic analysis of endogenous and exogenously expressed APP. a) Immunogold EM analysis of HeLa cells transfected with APP^{GFP} and labeled with an anti-GFP antibody. Arrowheads indicate APP^{GFP} inside the lumen of endosomal structures. Scale bar=0.3 µm. **b**) Confocal analysis of mouse hippocampal neurons (DIV14) labeled with antibodies to endogenous APP (APP-Cter) and the indicated antibodies. Pictures emphasize naturally occuring enlarged endolysosomal structures showing luminal APP cytodomain. Scale bar=0.4µm. **c and d**) Confocal analysis of HeLa cells transfected with APP^{RFP} and $Rab5_{Q79L}{}^{GFP}$ ($Rab5_{Q}{}^{GFP}$), and treated with 10µM cycloheximide (CHX) for 3h at 37°C. In (**c**), cells were then incubated for 20 min with an antibody to the N-terminal ectodomain of APP (22C11) at 4°C to label the cell surface pool of APP (left panel, confocal acquisition of basal plane; right panel, nuclear plane). In (**d**), cells were then submitted to a chase phase of 15 min (left panel) or 30 min (right panel) with medium at 37°C , prior to confocal analysis. Scale bar=10µm.



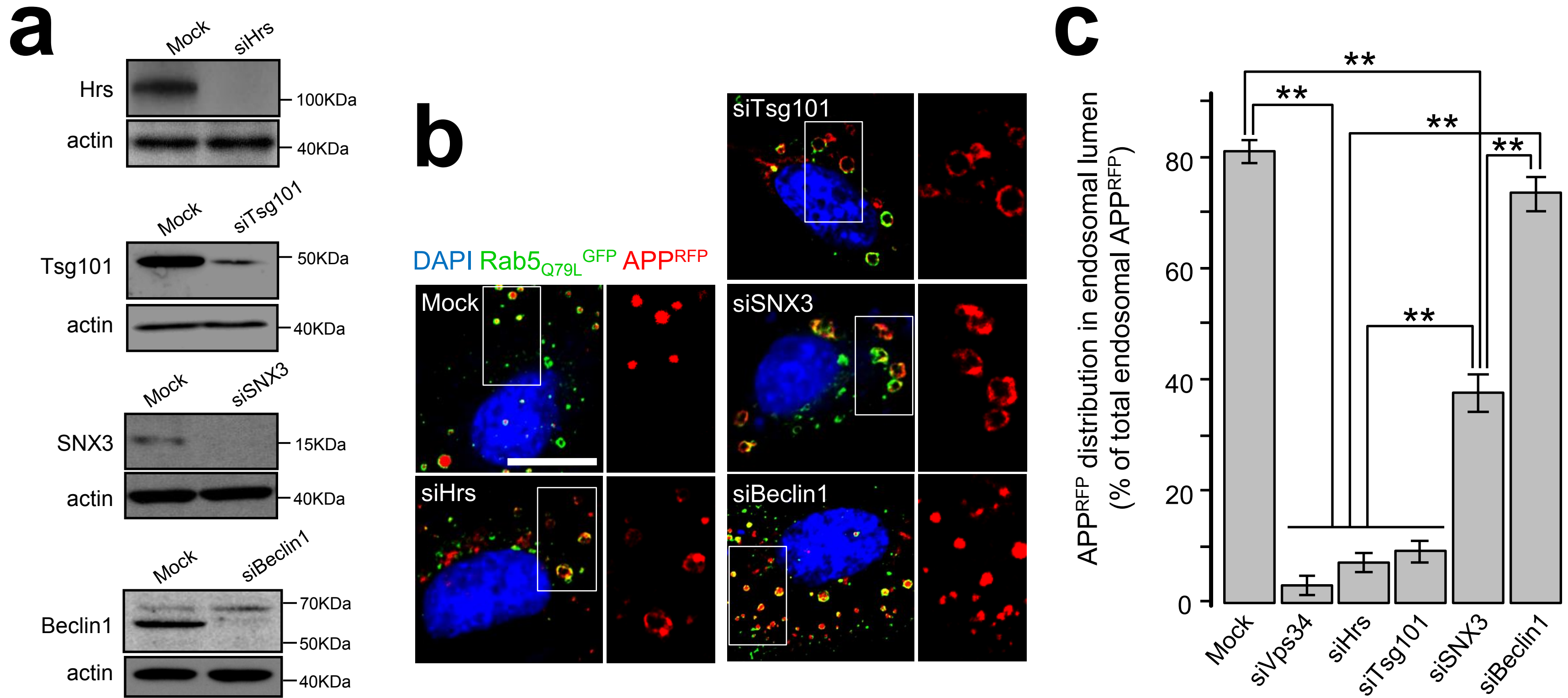
Supplementary Figure S5: Effect of wortmannin on PI3P and the endosomal pool of APP^{RFP}.

a) Confocal analysis of HeLa cells transfected with APP^{RFP} and FYVE-$FYVE^{GFP}$ and subjected to a treatment with vehicle (upper panel) or 100nM wortmannin (lower panel), prior to immunolabeling with the indicated antibodies. Scale bar = 10µm. **b**) Confocal analysis of HeLa cells transfected with APP^{RFP} and $Rab5_{Q79L}{}^{GFP}$ and treated with vehicle (MOCK) or 100nM wortmannin. Upper panel, confocal images showing mock-treated (left) and wortmannin-treated (right) cells. Scale bar = 5µm. Lower panel, quantification of the endosomal distribution of APP^{RFP} after wortmannin treatment, expressed as % of total APP^{RFP}: internal = inside endosomal lumen, peripheral = limiting membrane of the endosomes. Values denote means ± SEM (n = 10 cells from 3 experiments with an average quantification of 15 endosomes per cell); asterisks denote P values < 0.001 (from a Student's *t*-test).



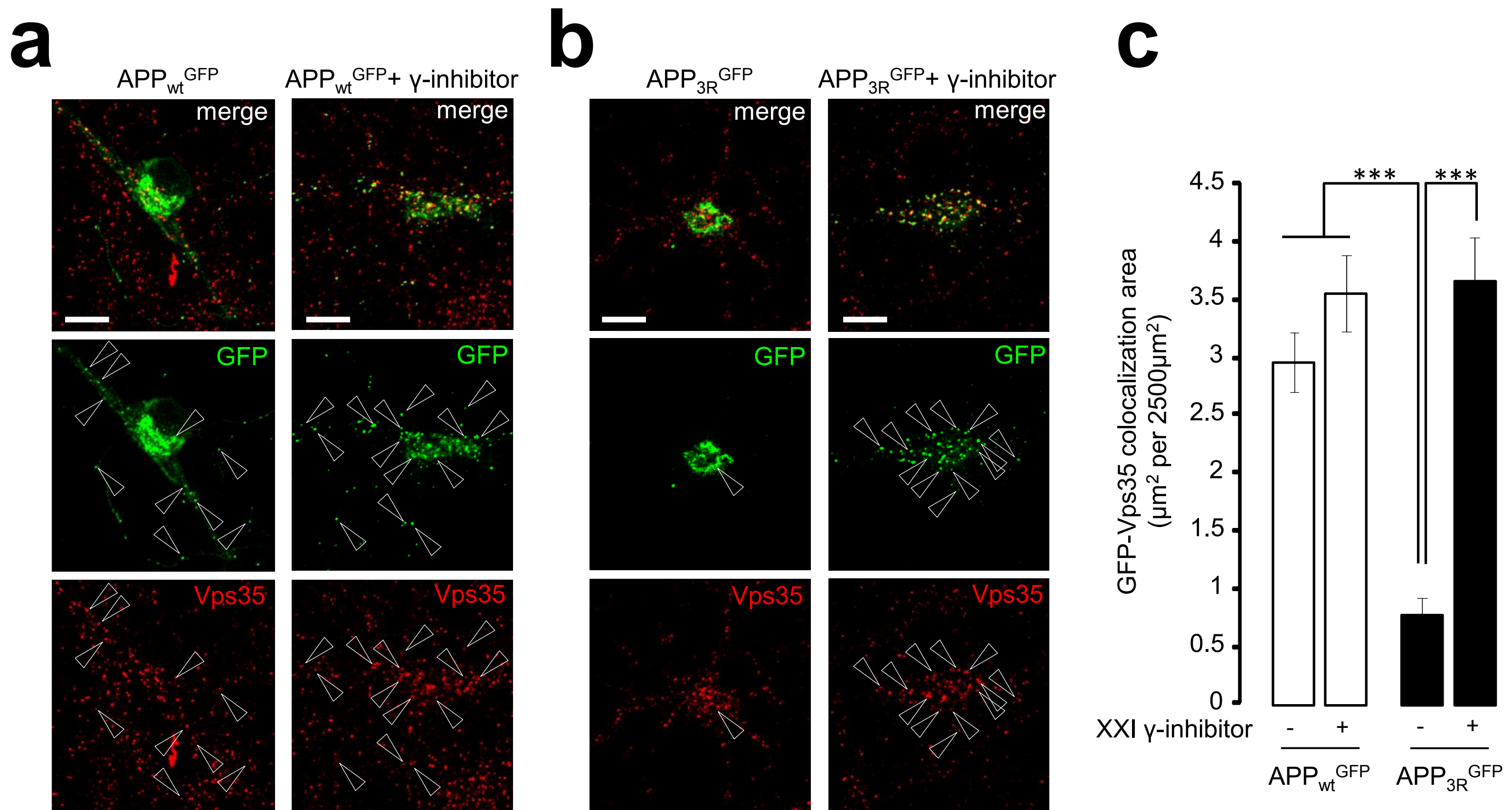
Supplementary Figure S6: Effect of Vps34 silencing on PI3P levels and the localization of endosomal markers.

a) Confocal analysis of HeLa cells either Mock-transfected (Mock) or transfected with siRNAs to Vps34 for 72h (siVps34), prior to labeling with a purified FYVE-FYVE-GST fusion protein and staining with a fluorescently-labeled anti-GST antibody and DAPI. Scale bar = 40μm. **b)** Quantification of the total FYVE-FYVE-GST fluorescence from cells shown in (a), expressed in arbitrary units (A.U.) (n=4); asterisks denote P values < 0.001 (from a Student's *t*-test). **c)** Confocal analysis of HeLa cells either Mock-transfected (Mock) or transfected with siRNAs to Vps34 for 72h (siVps34), followed by a second transfection with FYVE-FYVE^{GFP}. Arrowheads indicate FYVE-FYVE^{GFP} positive-cells. Scale bar = 60μm. **d)** Confocal analysis of HeLa cells processed as in (a), but labeled with antibodies to endosomal markers, EEA1 and Vps35. Scale bar = 20μm.



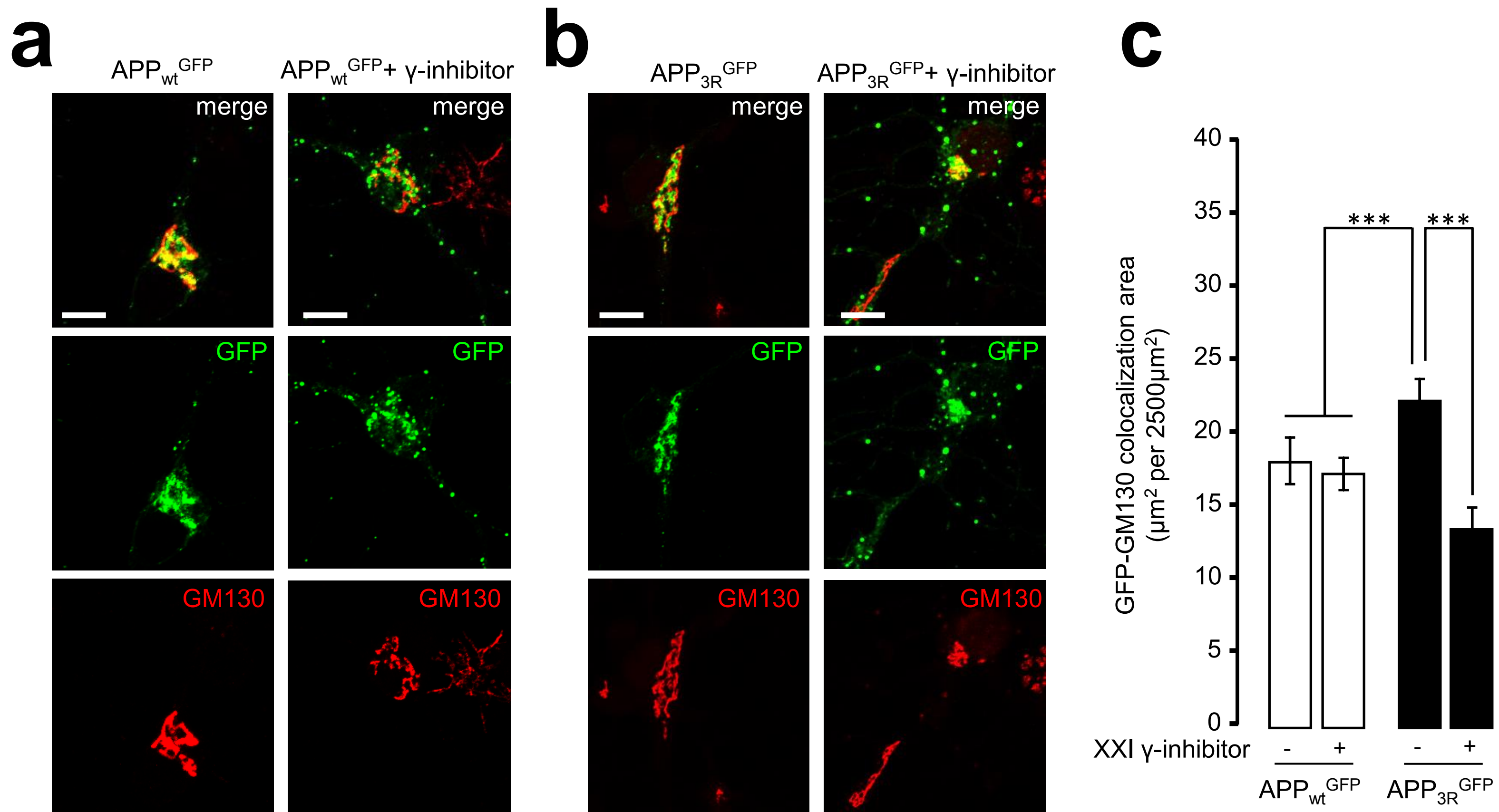
Supplementary Figure S7: Distribution of APP^{RFP} in Rab5$_{Q79L}$^{GFP} endosomes after silencing of Vps34, Hrs, Tsg101, SNX3 or Beclin1.

a) Representative Western blots showing decreased levels of Hrs, Tsg101, SNX3 and Beclin1 in the corresponding knockdowns after 72 h. Actin is used as loading marker. **b**) Confocal pictures from the Hrs, Tsg101, SNX3 and Beclin1 knockdowns showing the accumulation of APP^{RFP} on the limiting membrane of enlarged endosomes in the Hrs, Tsg101 and SNX3 knockdowns, in contrast to the Mock control and the Beclin1 knockdown, where the fluorescence of APP^{RFP} is predominantly luminal. Scale bar = 10μm. **c**) Quantification of the endosomal distribution of APP^{RFP} in HeLa cells after transfection for 72 h with siRNAs to Vps34, Hrs, Tsg101, SNX3 or Beclin1, followed by a second transfection with APP^{RFP} and Rab5$_{Q79L}$^{GFP} constructs. Cells were analyzed by confocal microscopy 24 h later. The localization of APP^{RFP} inside the endosomal lumen (internal) or on the endosomal limiting membrane (peripheral) was quantified and expressed as % of the total endosomal APP^{RFP}. Values denote means ± SEM (n=66, 43, 43, 31, 50, and 35 cells for the MOCK, Vps34, Hrs, Tsg101, SNX3 and Beclin1 knockdowns, respectively; from a pool of 5 experiments, with an average of approximately 15 endosomes per cell). Asterisks denote P values < 0.01 (**) (from a one-way ANOVA with post-hoc Tukey test).



Supplementary Figure S8: Subcellular distribution of the 3R APP mutant in primary neurons in relation to the Vps35-positive compartment.

Mouse hippocampal neurons were transfected with APP_{wt}^{GFP} (in **a**) or APP_{3R}^{GFP} (in **b**) at DIV9. After 24h in the presence (right panels) or absence (left panels) of γ-secretase inhibitor XXI, neurons were stained with an anti-Vps35 antibody (red) and imaged with confocal microscopy. Scale bar = 10 μm. Arrowheads indicate areas of colocalization between APP^{GFP} and Vps35. **c**) Quantification of the colocalization between APP^{GFP} and Vps35 measured in colocalization area per 2500μm^2 image area. Values denote means ± SEM (n = 26 cells); asterisks denote P values < 0.001 (from a Student's *t*-test).



Supplementary Figure S9: Subcellular distribution of the 3R mutant in primary neurons in relation to the GM130-positive compartment.

Mouse hippocampal neurons were transfected with APP_{wt}^{GFP} (in **a**) or APP_{3R}^{GFP} (in **b**) at DIV9. After 24h in the presence (right panels) or absence (left panels) of γ-secretase inhibitor XXI, neurons were stained with an anti-GM130 antibody (red) and imaged with confocal microscopy. Scale bar = 10 µm. Arrowheads indicate areas of colocalization between APP^{GFP} and GM130. **c**) Quantification of the colocalization between APP^{GFP} and GM130 measured in colocalization area per 2500µm^2 image area. Values denote means ± SEM (n = 37 cells); Asterisks denote P values < 0.001 (from a Student's *t*-test).

FIGURE 2a

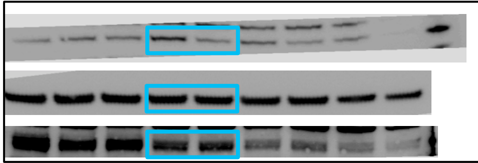


FIGURE 2d

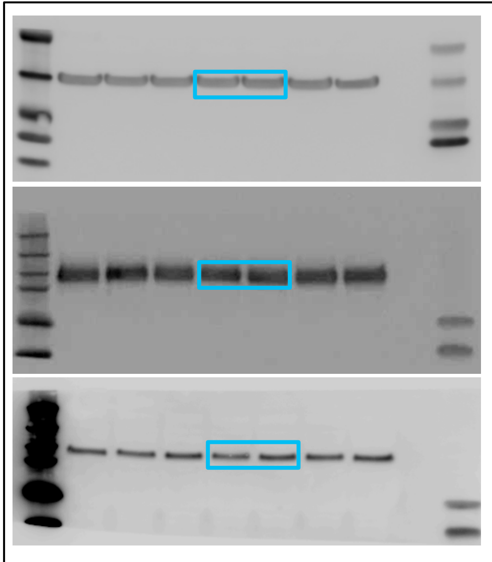


FIGURE 4h

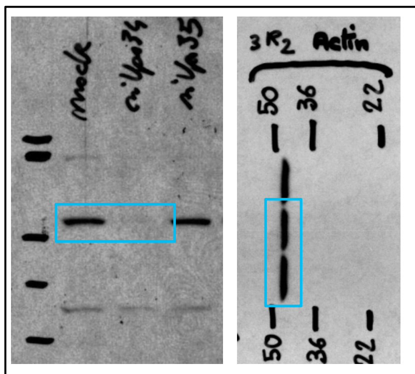


FIGURE 5b

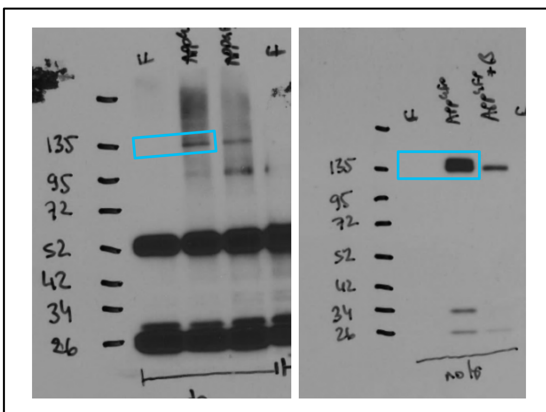


FIGURE 5a

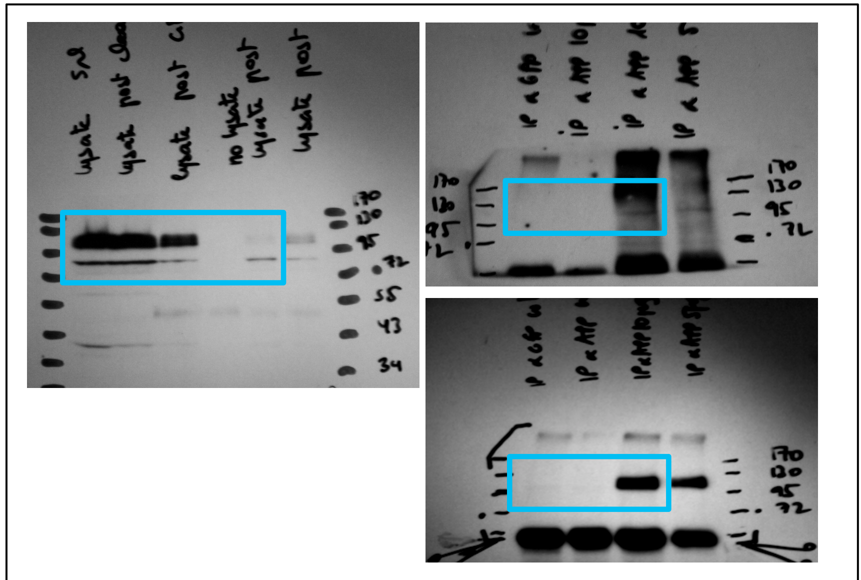


FIGURE 5d

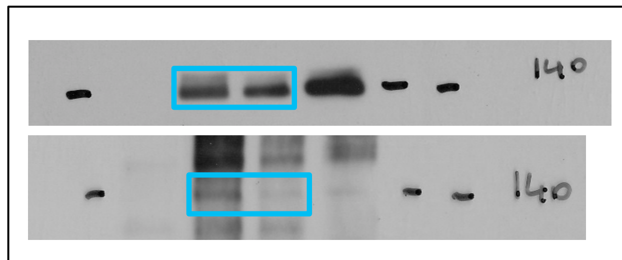
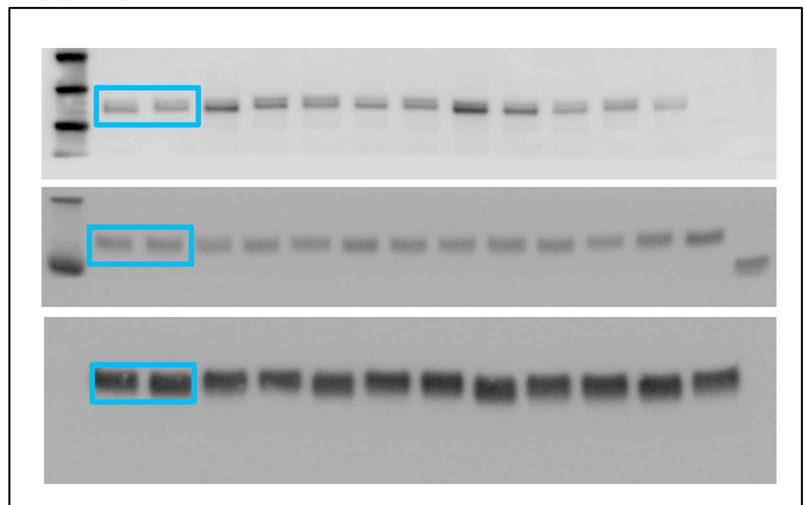


FIGURE 8



Supplementary Figure S10: Full scans or digital pictures of immuno-blots shown in figures 1-8