Supplemental Experimental Procedures

Cell Culture, Generation of Stable Cell Lines and Primary Hippocampal Neuron Culture
All culture media and sera are from Life Technologies. Mouse embryonic fibroblasts (MEFs), wild-type (WT), PSEN1 and PSEN2 knock-out (dKO, (Herreman et al., 2000)), rescued dKO cell lines, HEK293, HEK293T, HeLa, A549, MCF7, MDA_MB, T24 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal calf serum (FCS). LNCaP, 22rv1, H2170 and LIM215 were grown in RPMI-1640 supplemented with 10% FCS. A549, MCF7, MDA_MB, T24, LNCaP, 22rv1, H2170, LIM215 cells were kindly provided by J. Swinnen, KU Leuven, Belgium. The highly pigmented human melanoma MNT-1 cells were cultured in DMEM/Glutamax, with 10% AIM-V medium, 20% FBS, 1% non-essential amino acids (Raposo et al., 2001). All cell lines were maintained in a humidified chamber with 5% CO_2 at 37°C. Primary hippocampal neuron cultures are derived from E17-E18 mouse or rat embryos (Esselens et al., 2004). Stable rescued dKO cell lines were generated from MEFs dKO using replication-defective recombinant retroviral system (pMCSV vector, Clontech) expressing the gene of interest. Patient primary fibroblast cells were obtained from Coriell Cell Repositories: three PSEN1 FAD A246E: A246E-1(AG06840), A246E-2 (AG06848), A246E-3 (AG08711), three control: Ctrl 1 (AG07871), Ctrl 2 (AG08509), Ctrl 3 (AG08701) and one PSEN2 FAD-N141I (AG09908). These cells were cultured in DMEM/L-Glutamine with 15%FBS.

Antibodies
The following monoclonal antibodies (mAbs) were commercially obtained: rat anti-LAMP1 (sc-19992, Santa Cruz); mouse anti-LAMP1 (BD Bioscience); mouse anti-transferrin receptor (TfR, clone H68.4, Life Technologies), anti-β-actin (AC15, Sigma), anti-tubulin (DMIA, Sigma), anti-AP1G1 (clone 88, BD Bioscience), anti-AP3D1 (clone 18, BD Biosciences), anti-Tau-1 (Clone PC1C6, Chemicon); rabbit anti-AP2M1 (ab75995, abcam), human Aβ AA 4-10 (WO2, the Genetics Company) and AA 1-16 (6E10, Signet Laboratories). The following polyclonal antibodies (pAbs) were purchased: rabbit anti-Rab11 (life Technologies), rabbit anti-PSEN1 (ab24748, abcam), rabbit anti-PSEN2 (ab51249, abcam), rabbit anti-Pen2 (ab18189, abcam) rabbit anti-AP1B1/AP2B1 (ab21981, abcam), rabbit anti-GFP (for immunoprecipitation, A-11122, Life Technologies), goat anti-GFP (for Western blot, 600-101-215, Rockland), chicken anti-GFP (for immunofluorescence, GFP-1020, aves Labs), rabbit anti-MAP2 (sc-20172, Santa Cruz), chicken anti-MAP2 (ab5392, abcam) rabbit anti-TRP1 (sc-25543, Santa Cruz), rabbit anti-PACS1 (ab56072, abcam), rabbit anti-TGN46 (ab16059, abcam), rabbit anti-EEA1 (sc-33585, Santa Cruz), rabbit anti-Notch1 (D1E11, Cell Signaling), rabbit-anti cleaved Notch1 (Val1744, Cell Signaling). The following antibodies were generously provided: mAb to Rab5 by (R. Jahn, MPI, Göttingen), pAb against Rab7 (P. Chavrier, CNRS, Paris), pAb against Ribophorin (R. Schekman, Berkeley, US), pAb against Cathepsin D (P. Saftig, Institut of Biochemistry, Kiel). Rabbit pAb against APP C-terminus (B63.3), PSEN1-NTF (B19.3), PSEN2-CTF (B24), PEN-2 (B126.1) and mAb against NCT (9C3) were generated in-house (Annaert et al., 1999; Esselens et al., 2004; Herreman et al., 2003). Affinity purified anti-peptide antibody recognizing the C-terminus of PMEL was previously described (Raposo et al., 2001). ELISA antibodies were obtained through collaboration with Janssen Pharmaceutica NV, Beerse, Belgium: JRF/cAb40/28 for Aβ1-40, JRF/cAb42/26 for Aβ1-42, and detection antibody JRF/AbN/25 against the N terminus of Aβ.

Plasmids, Transfection, RNAi
The plasmids pSG5-C99-3xFLAG and pcDNA-APPsw (Swedish mutant of APP) were previously described (Bentahir et al., 2006; Sannerud et al., 2011) respectively. cDNAs encoding human WT PSEN1 and PSEN2 were cloned into pEGFP-C1 between EcoRI and BamHI restriction sites. GFP-PSEN1 and GFP-PSEN2 sequences were digested with AfeI and HpaI, and inserted into the HpaI linearized pMSCV-puro (Clontech). pTagRFP-PSEN2 were obtained (Evrogen). For lentivirus infection of neurons, EGFP-PSEN1 and EGFP-PSEN2 inserts were cut out from pEGFP-PSEN1-C1 and pEGFP-PSEN2-C1 using NheI/XbaI restriction sites and ligated into a lentiviral plasmid pFUGW cut with XbaI. cDNAs encoding GFP fused to PSEN1 and PSEN2 hybrids(Zhao et al., 2008) were cloned into pMSCV for virus production.). The hybrids were named as such: Hyb. 1: (1-268(PSEN1), 275-304(PSEN2), 299-467(PSEN1); hyb. 2: (1-298(PSEN1), 305-355(PSEN2), 375-467(PSEN1)); hyb. 3: (1-298(PSEN1), 305-448(PSEN2)); hyb. 4: (1-76(PSEN2), 71-467(PSEN1)); hyb. 5: (1-70(PSEN1), 77-448(PSEN2)). GST-N_{1-81}PSEN1-NTF and GST-N_{1-86}PSEN2 were generated previously (Annaert et al., 2001). Mutations in PSEN2 were introduced using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer with the forward primers: S19A: 5'-gtgatgagcggacggccctaatgtcggcc-3'; S19D: 5'-gtgtgatgagcggacggacctaatgtcggccgag-3'; S7A-S9A (named SSAA) 5'-ctcacattcatggccgctgacgccgaggaagaagtgtgtg-3'; S7D-S9D (named SSDD): 5'-ctcacattcatggccgatgacgacgaggaagaagtgtgtg-3'; E16A-L20A-M20A (named AxxxAA): 5'-gtgtgtgatgcgcggacgtccgcagcgtcggccgagagcccc-3' and there reverse complement. Mutations in GST-N_{1-86}PSEN2 were generated using the following primers: S19A: 5'-gtgatgagcggacggccctaatgtcggcc-3'; S19D: 5'-gtgtgatgagcggacggacctaatgtcggccgag-3'; S7A-S9A (named SSAA) 5'- tggtgacattcatggccgctgacgccgaggaagaagtgtgtg-3'; S7D-S9D (named SSDD): 5'- caccatggtgacattcatggccgatgacgacgaggaagaagtgtgtgatgag-3'; E16A (named AxxxLM): 5'-gaagaggtgtgtgatgcgcggacgtccttgatg-3'; M21A (named ExxxLA): 5'- agcggacgtccttggcgtcagccgagagcc-3'; E16A-L20A-M20A (named AxxxAA): 5'-agaggtgtgtgatgcgcggacgtccgcggcgtcagccgagagcc-3' and there reverse complement. PSEN1-FAD mutations were generated using the following primers: Y115H: 5'-gatgggcagctaatccataccccattcac-3';

M139V: 5'-gaatgctgccatcgtgatcagtgtcattg-3'; L166P: 5'-ggtcatccatgcctggcctattatatcatctc-3' I213T: 5'-gggaatgatttccactcactggaaaggtcc-3'; G384A: 5'-cttggattggcagatttcattttctacagtgttctgg-3'; and there reverse complement. PSEN2-FAD mutations were generated using the following primers: T122P: 5'-gacagctcatctacccgccattcactgag-3'; N141I: 5'-caactccgtgctgatcaccctcatcatg-3'; M239V: 5'-cagtgcgctcgtggccctagtgttc-3'; M239I: 5'-gatcagtgcgctcattgccctagtgttc-3'; and there reverse complement. All constructs were verified by sequencing.
Cells were transfected with FugeneHD (Roche Diagnostics) to express C99-3xFLAG. Primary hippocampal neurons were infected at 3 to 4 days in vitro (DIV) and analyzed at 7 DIV.
The siRNAs targeting human PSEN1 (L-004998), human PSEN2 (L-006018) and non-targeting control (D-001810) ON-TARGETplus SMARTpool were from Thermo Scientific. SiRNA transfections were performed using JetPRIME (Polyplus transfections) and analyzed 72h after down-regulation.
Plasmid to express LAMP1-mCherry is from addgene (#45147).

Virus Production
For virus production, HEK293T cells were transiently transfected using FuGENE6 (Promega) according to the manufacturer instruction. For retrovirus packaging, pMSCV expressing the gene of interest was cotransfected with the helper plasmid pIk (Ecopac). Lentiviral particles were produced by co-transfection of appropriate lentiviral vectors with packaging (pCMV-ΔR8.74) and envelope (pMD2.G) plasmids in HEK293T cells. Plasmids pFUGW, pCMV-ΔR8.74 and pMD2.G were a gift of Y. Arsenijevic (University of Lausanne, Jules-Gonin Eye Hospital, Lausanne, Switzerland). Lentiviral Particles were purified by ultracentrifugation and resuspended in DMEM/F12 medium.
For transduction, viral particles were diluted in medium containing Polybrene (8 ng/µl, Sigma). After 24 h, medium was refreshed. To establish stable cell lines, transduced cells were selected using 5 µg/ml puromycin (Sigma).

Western blot
Protein concentrations were determined by the Bio-Rad DC protein assay (Bio-Rad). Samples were separated by SDS-PAGE (4-12% Bis-Tris NuPAGE gels in MES running buffer (Life Technologies) and transferred onto nitrocellulose membranes (Life Technologies). After blocking in 5% non-fat milk, membranes were incubated with primary antibody (4°C, overnight) followed by washing and incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1h, room temperature). After final washing, immunodetection was done using enhanced chemiluminescence (Western Lightning-Plus ECL, PerkinElmer), and immunoreactive protein bands were digitally captured and quantified on a Fuji MiniLAS 3000 imager (Fuji, Düsseldorf, Germany) using Aida Image Analyzer software (Raytest, Germany). Data are represented as mean ± SEM of at least three independent experiments.

Discontinuous Sucrose/D_2O Flotation Gradient
For each gradient, two 600cm^2 tissue culture plates of confluent MEFs were used. All steps were carried out at 4°C and performed as described (Gorvel et al., 1991) with some modifications. Briefly, cells were scraped in ice-cold PBS Dulbecco's and centrifuged (5 min, 650xg). Cell pellets were resuspended in 2 ml homogenization buffer (250 mM sucrose, 3 mM imidazole, pH=7.4), and homogenized using a cell cracker (4 passages, clearance 10 µm, Isobiotec, Germany). Protease inhibitors (PI, cOmplet Protease Inhibitor Cocktail tablet from Roche) were added and after a brief centifugation (10 min at 800xg) a post-nuclear supernatant (PNS) was brought to 40.6% sucrose and loaded on the bottom of a centrifugation tube and overlaid with 3,7 ml 16% sucrose, 3 ml 10% sucrose (in D_20, 3 mM imidazole, pH=7.4, 0.5 mM EDTA) and finally 1.5 ml homogenization buffer. After centrifugation (1.4h, at 151,263xg) in an SW41 rotor, twelve 1ml fractions were collected and equal amounts of protein were used for Western blotting and γ-secretase assays.

Isolation of Late Endosomes/Lysosomes (LE/LYS)
LE/LYS were isolated according to (Tharkeshwar et al., submitted). Cells (90% confluency) were incubated (Pulse) with DMSA-coated SPIONs suspended in culture medium (concentration 0.2 mg/ml) for 15 min at 37°C. After a wash with PBS, cells were re-incubated (Chase) at 37°C in fresh medium for 240 min, followed by three successive washes with acidic buffer (0.15M glycine, pH 3), cold PBS and harvested by scraping, centrifugation (180 g, 10 min) and resuspension in homogenization buffer (HB; 250 mM sucrose, 5 mM Tris and 1 mM EGTA pH 7.4 supplemented with PI). After cell cracking (12 passages, clearance 10µm) the total homogenate was centrifuged (800xg, 10 min) and the PNS loaded on a LS column (pre-equilibrated with HB) placed in a strong magnetic field (0.5 T) (SuperMACSII, Miltenyi). The hydrophilic matrix of the LS column enhances retainment of SPIONs along with its adhering subcellular compartments, allowing nonmagnetic material to be removed by extensive washes with ice-cold HB. Next, the column was detached from the magnetic field and the bound fraction containing LE/LYS was eluted using HB, centrifuged (126,000xg, 1 h) and the resulting pellet re-suspended in 200 µl HB. Equal amounts of protein were denatured in sample buffer (Life Technologies), and analyzed by Western blot. Each experiment was performed two to three times.

Cell Surface Biotinylation
Cell surface biotinylation was performed as described (Sannerud et al., 2011). Briefly, cells were placed on ice, washed in PBS (pH=8) and incubated in PBS (pH=8) supplemented with 0.25mg/ml sulfo-NHS-SS-Biotin (Pierce) (10min at 4°C). Excess of biotin was washed out and remaining biotin quenched with 1% BSA in PBS (15min at 4°C). Cells were lysed in lysis buffer (50mM HEPES, pH 7.2, 100mM NaCl, 1% Triton X-100, with PI). Total protein was measured and

biotinylated proteins were pulled down from equal amounts of extracts using streptavidin Sepharose beads (Pierce) (4°C, 2h on a rotation wheel). After washing, bound material was eluted from the beads using 2x loading buffer (Life Technologies) containing 2% β-mercaptoethanol (70°C for 10 min), and analyzed by Western blot. Each experiment was performed at least three times. Values are presented as mean ± SEM. β. P values were determined by Student's *t*-test, unpaired, unequal variance (*$p<0.05$, *$p<0.01$, ***$p<0.001$).

Co-Immunoprecipitation (Co-IP)

Cells were harvested in ice cold PBS+/+. After centrifugation (1,700xg for 5 min, 4°C) cell pellets were resuspended in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% CHAPSO, PI). After 1 h at 4°C, lysates were centrifuged (20,000xg, 15 min. 4°C) and total protein was measured from the supernatant. The same amount of protein (500 µg) was incubated with 1.5 µl of rabbit anti-GFP, or rabbit anti-PSEN2 for 2 h at 4°C, followed by incubation with protein-G (20 µl) overnight at 4°C, under constant rotation. The immune complexes were washed three times with lysis buffer, once with 0.5x Tris-buffered saline (TBS, 1x: 10 mMTris, 150 mN NaCl, pH =7.4), then denatured in 18 µl of sample buffer and analyzed by Western blot. Total lysate (20 µg) was used as input. Blots were probed as indicated in the Figures. β. Each experiment was performed at least 3 times. Values are presented as mean ± SEM. P values were determined by Student's *t*-test, unpaired, unequal variance (*$p<0.05$, *$p<0.01$, ***$p<0.001$).

Aβ detection

Cells were processed 24 h after transfection with C99-3xFLAG (or APPsw). Conditioned medium were collected and cell debris were removed by centrifugation. Cells were washed twice with PBS+/+ and lysed in lysis buffer (50 mM Tris-HCL, pH 7.4; 150 mM NaCl, 1% Triton X-100; 0.1% SDS; 0.5% sodium deoxycholate; PI).

For total Aβ detection:

Equal volume of conditioned medium (3% of total volume, Aβ extracellular pool) and equal amount of total protein (10 µg, Aβ intracellular pool) from cell lysate were analyzed by westernblot. For each cell line, Aβ signals (extracellular and intracellular) were quantified and corrected for C99 expression level.

For Aβ40 and 42 quantification, ELISA:

Aβ40 and 42 were measured in conditioned medium (3% of total volume, extracellular pool) and in cell lysate (10 µg, intracellular pool) by ELISA. 96-well MSD plates were coated with anti-Aβ40 or -Aβ42 antibodies overnight. After rinsing 5x with washing buffer (PBS +0.05% Tween-20), plates were blocked with 150 µl/well 0.1% casein buffer for 1 h at room temperature (600 rpm) and rinsed 5x with 150 µl/well washing buffer. 25 µl SULFO-TAG JRF/AbN/25 detection antibody diluted in blocking buffer was mixed with 25 µl of standards (synthetic human Aβ1-40, and Aβ1-42 peptides) or reaction samples diluted in blocking buffer and loaded 50 µl per well. After overnight incubation at 4°C, plates were rinsed with washing buffer and 150 µl/well of the 2× MSD Read Buffer T (Tris-based buffer containing tripropylamine, purchased from Meso Scale Discovery) was added. Plates were immediately read on a Sector Imager 6000 (Meso Scale Discovery). We determined the amount for Aβ40, and Aβ42 and corrected it for the C99 expression level and normalized to PSEN1 or PSEN2 as indicated. Ratio 42/40 were normalized to PSEN1 or PSEN2 as indicated, but to the secreted pool in order to compare secreted versus cellular pool of Aβ. Each experiment was performed at least 3-6 times. Values are presented as mean ± SEM. P values were determined by Student's *t*-test, unpaired, unequal variance (*$p<0.05$, *$p<0.01$, ***$p<0.001$).

GST-Assay

The cDNAs encoding GST fused to the first 81 and first 86 amino acids of PSEN1 and PSEN2, respectively, were subcloned into pGEX4T-1, transformed in E. coli BL21. Recombinant GST fusion proteins were induced using 0.1mM IPTG for 2 h at 30°C and extracted and purified as described (Annaert et al., 2001). Briefly, E. coli pellets were resuspended in Tris-saline buffer (150 mM NaCl, 10 mM Tris, pH 7.4), sonicated and further extracted with Triton X100 (15 min). The cleared extracts (20 min, 16,000xg) were mixed with glutathione coupled to Sepharose (GE healthcare) 30 min, 4°C). After extensive washing, beads were incubated overnight with extracts of brain cortexes of C57 black p1 pups. Extraction of cortexes was performed in PBS-/-, 1% Triton X-100 supplemented with PI. The next morning beads were washed 4x in PBS-/- with 1% Triton X-100 and PI and once with 0.3x TBS. Bound material was eluted with 4x sample buffer and processed for SDS-PAGE and Western blot.

Notch ligand stimulation and immunoblotting

Cells were plated on rDll4-Fc (1µg/ml; R&D Systems) coated plates and treated with DMSO or Dibenzazepine (DBZ; 0.2 µM; Syncom, Groningen, The Netherlands) for 16 h. Cells were directly lysed in Laemmli buffer prior to resolving by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and blocked for 1 h in 5 % skim milk in PBS, 0.05 % Tween-20 (PBS-T). Membranes were probed overnight at 4°C with primary antibodies and bound antibodies were visualized using HRP-linked secondary antibodies (Cell-Signaling) and ECL Luminescence (Pierce Biotechnology). Anti-Notch1 antibody (D1E11, 1:1000), anti-NOTCH2 antibody (D76A6, 1:2000), anti-cleaved Notch1-Val1744 antibody (D3B8, 1:1000) and anti-Presenilin 2 antibody (2192, 1:1000) were purchased from Cell signaling. Anti-Presenilin 1 antibody (1:1000) was purchased from Chemicon and anti-β-actin antibody was purchased from MP Biomedicals.

Yeast three-hybrid
DNA constructs: BamH1/PstI fragments encoding either wild-type, E16A, S19A, S19D, M21A or E16A/L20A/M21A mouse PSEN2 cytosolic tail (residues 1-87) were amplified from the corresponding constructs in pGEX-4T1. These fragments were subcloned into the BamH1/PstI sites of the multiple cloning site (MCS) 1 of pBridge (Clontech) vector-based constructs containing cDNAs encoding either human σ1A, rat σ2 or human σ3A in their MCS2 (generating pBridge-PSEN2 tail.σ1A, pBridge-PSEN2 tail.σ2 and pBridge-PSEN2 tail.σ3A plasmids). The pBridge-PSEN tail M21L.σ1A, pBridge-PSEN tail M21L.σ2 and pBridge-PSEN tail M21L.σ3A plasmids were generated by site-directed mutagenesis (QuickChange, Agilent) of the wild-type constructs. All constructs were verified by sequencing. The pGADT7 (Clontech) vector-based constructs encoding mouse AP-1 γ1, rat AP-2 αC or human AP-3 δ subunits were previously described (Janvier et al., 2003; Mattera et al., 2011).
Yeast three-hybrid (Y3H) analysis: Assays were performed using the HF7c reporter strain as described (Janvier et al., 2003; Mattera et al., 2011). Double transformants were selected in medium lacking leucine, tryptophan and methionine but containing histidine (+His), while interactions were assessed in the same medium lacking histidine (-His). The positive and negative controls used in the assays are described in the corresponding figure legend.

γ-Secretase Activity Assay
Assays using recombinant substrate (Figure 1). Equal amount of total protein (7.5μg) of each fraction was resuspended in 15 μl TE buffer (5 mM Tris-HCl, 1 mM EDTA, pH=7.0) containing 0.5%CHAPS and incubated for 1 h at 4°C. Extracts were incubated overnight at 37°C with 1 μl recombinant APP-C100-FLAG (Li et al., 2000), 1 μl DMSO and 1 μl 1M Tris-HCl pH=7.0. De novo formed Aβ was analyzed by Western blot (WO-2 mAb).
Assay with endogenous substrates (Figure 5 and 6). All procedures were carried out at 4°C. Cells (80% confluent) were washed twice with ice cold PBS and scraped. After centrifugation (600xg, 5 min), cells were resuspended in 750 μl hypotonic buffer (10 mM Tris pH 7.6, 1 mM EDTA, 1 mM EGTA) supplemented with PI and incubated on ice for 15 min. After centrifugation (20,000xg, 10 min) to remove cell debris, protein concentration of the supernatant was measured and four aliquots of 60 μg were again centrifuged (20,000xg, 60 min). Pellets were resuspended into 15 μl citrate buffer (150 mM sodium citrate, pH=6.4); three samples, from which one was supplemented with 1 μM DAPT (control), were incubated at 37°C for 3 h, and one was kept at 4°C (starting material). The assay was stopped after three h by adding 5 μl of 4x loading buffer followed by heating (10 min, 70°C), loaded for SDS-PAGE and analyzed by Western blot. Proteolytic activity was quantified using a C-terminal antibody washed in PBS (pH=8) and incubated in PBS (pH=8) supplemented with 0.25 mg/ml sulfo-NHS-SS-Biotin (Pierce) (10min at 4°C). Excess of biotin was washed out and remaining biotin quenched with 1% BSA in PBS (15min at 4°C). Cells were lysed in lysis buffer (50mM HEPES, pH 7.2, 100 mM NaCl, 1% Triton X-100, with PI). Total protein was measured and biotinylated proteins were pulled down from equal amounts of extracts using streptavidin Sepharose beads (Pierce) (4°C, 2h on a rotation wheel). After washing, bound material was eluted from the beads using 2x loading buffer (Life Technologies) containing 2% β-mercaptoethanol (70°C for 10 min), and analyzed by Western blot. Each experiment was performed at least 3 times. Values are presented as mean ± SEM. P values were determined by Student's *t*-test, unpaired, unequal variance (**p*<0.05, **p*<0.01, ****p*<0.001).

Mouse brain section
Brain sections were prepared as described in Rubio et al (Rubio et al., 2012). Briefly, CD-1 mice (P10 days) were deeply anesthetized and perfused with 4% paraformaldehyde in phosphate buffer. The brains were cryoprotected, frozen, and 30-μm sections were cut. Coronal sections were stored in a cryoprotectant solution (30% glycerin, 30% ethylene glycol, and 40% phosphate buffer) at −20°C until use.
Immunohistochemistry:
Tissue specimens were permeabilized in PBS+/+ containing 0.2% Triton X-100 and then incubated in blocking buffer (10% goat serum, 0.2 M Glycine, 0.2% Triton-X100,0.2% gelatin, in PBS+/+) for 2h. Primary antibodies, diluted in blocking buffer were incubated with the specimens overnight at 4C. After few washes in PBS containing 0.2% Triton X-100, sections were incubated with secondary antibodies diluted in blocking buffer, for 1h. After few washes, sections were mounted in Mowiol.

Confocal Laser Scanning Microscopy and Quantification
Cells were plated on glass coverslips, transfected 24 h later and processed for indirect immunelabeling the next day (Sannerud et al., 2011). Images were captured on a confocal microscope (Leica TCS SP5 II, Leica Microsystems) connected to an upright microscope, using an oil-immersion plan Apo 60x A/1.40 NA objective lens. Image acquisition was performed with LAS (Leica Microsystems). For live imaging of neurons treated with LysoTracker Red DND-99 (50 mM, Thermo Fisher Scientific), images were captured at 37°C on a Nikon A1R confocal system connected to an inverse microscope (Ti-2000; Nikon) using an oil-immersion Plan-Apochromat 60× A/1.40 NA objective. Data were collected using Nikon Imaging Software and further processed with ImageJ and PhotoshopCS6 (Adobe, CA).
ImageJ software with the plugin JACoP (Bolte and Cordelieres, 2006) was used for signal overlap quantification (Mander's coefficient). Images were acquired with identical settings and regions of interest (ROI) covering whole cells were used for quantification. Data are represented as Mean ± SEM for 8 to 15 cells in three independent experiments). Dendrite/axon polarity index (D/A) of PSEN1, PSEN2 WT and mutants (AxxxAA, S19D and S19A, Figure 6C') were calculated in neurons expressing PSEN1 or -2 and immunostained for MAP2 (to label dendrites) and Tau1 (to label

axons). Images were acquired with identical settings. Several ROIs were drawn along neurites (dendrites or axons) within a field of view containing one or two neurons expressing PSEN1 or -2. For each ROI Mander's coefficients were measured for GFP:MAP2 and GFP:tau1. For each ROI the polarity index corresponding to the ratio GFP:MAP2 /GFP:tau1 was calculated and represented as mean ± SEM. P values were determined by Student's *t*-test, unpaired, unequal variance (*$p<0.05$, *$p<0.01$, ***$p<0.001$).

Electron microscopy

For conventional EM of MNT-1 cells, cells grown on coverslips were processed for Epon embedding and ultrathin sections and then contrasted with uranyl acetate and lead citrate as described previously (Raposo et al., 2001).
For conventional EM analysis of RPE sections, tissue blocks (eyes) from Psen2−/− mice (maybe you need to describe more, background and age for example) and WT mice were processed for Epon embedding and ultrathin sections and then contrasted with uranyl acetate and lead citrate as described previously (Lopes et al., 2007).
All samples were analyzed by using a FEI Tecnai Spirit electron microscope (FEI Company), and digital acquisitions were made with a numeric camera (Quemesa; Soft Imaging System).
Image Analysis and Quantification. Melanosome stages were defined by morphology (Raposo et al., 2001; Seiji et al., 1963). Quantification of melanosomes was determined by using iTEM software (Soft Imaging System).

Correlated light and electron microscopy (CLEM)

The MEF dKO rescued with GFP-PSEN2-WT or GFP-PSEN2-S_{19}A were plated on glass bottom dishes (MatTek). Cells were either expressing LAMP1-mCherry (24h transcient expression) or labeled with MitoTracker (100 nM, 30 min, Thermo Fisher Scientific). Cells were fixed first by adding 1 ml pre-warmed double strength fixative solution (7% paraformaldehyde, 0,4% glutaraldehyde in 0,1 M phosphate buffer) to the medium while rotating at room temperature. After 10 min solution was replaced by single strength fixative solution (4% paraformaldehyde, 0,2% GA in 0,1M phosphate buffer). Cells were kept in this fixative solution in the dark until imaging (up to 1 h).
Super resolution light microscopy:
Structured illumination microscopy was used as a technique for super resolution light microscopy. An inverted Zeiss Elyra equipped with a stage for mounting dishes was used. During SIM imaging the cells were maintained in PBS -/-. To be able to correlate the same cells in light and electron microscopy, two marks were drawn on the bottom of the glass coverslips with a distance of 5 mm.
Nicely spread single cells, expressing GFP-PSEN2 (WT or S19A mutant) together with LAMP1-mCherry or MitoTracker, near one mark, were identified. Once found a cell of interest both transmitted light and fluorescence z-stack SIM images were taking using the 63x objective. The z-stack slices have a 0.2 µm thickness. After light imaging, the PBS -/- was replaced by a fixative solution of 2.5% GA in 0.1 M sodium cacodylate buffer. The cells were kept in this fixative solution overnight at 4°C.
Preparation for TEM:
Cells were washed three times in 0.1 M sodium cacodylate buffer. To enhance EM membrane contrast, cells were first treated with 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, next with 1 % tannic acid dissolved in 0.1 M cacodylate buffer for 30 min, followed by 1 % osmium tetroxide in 0.1M cacodylate buffer for 30 min. All steps were done at room temperature with 0.1 M sodium cacodylate washing steps in between.
The cells were then dehydrated in solutions of ethanol at increasing concentrations of 30%, 50% and 70% at 4°C for 5 min on a rotator. Subsequently, cells were stained *en bloc* with 3% uranyl acetate in 70% ethanol for 30 min at 4°C in the dark followed by further dehydration with increasing concentrations of 90% and 100% ethanol (3x) at 4°C for 5 min on a rotator. After dehydration, the cells were infiltrated with resin (Agar 100)/ethanol mixtures. The next day, the cells were embedded in Agar 100 in inverted BEEM-capsules and cured for 2 days at 60°C.
After polymerization the glass coverslip was removed from the dish by alternatingly placing the dishes on a heating plate of 40°C and immersing them in liquid nitrogen. Once the glass coverslip was removed, the cells were visible in the resin blocks. With a stereomicroscope, the cells of interest were located by matching them with the overview images taken with the SIM, and marked by a surrounding square using a scalpel. Next, after trimming the block, 50 nm serial ultrathin sections were cut from the marked block surface using a Reichardt Ultracut E ultramicrotome. All sections were collected as ribbons of 4-5 sections on triple slot grids (Ted Pella). Every grid was then poststained with 3% uranyl acetate in water for 10 min and Reynold's lead citrate for 2 min.
Transmission electron microscope:
EM images were taken by a JEOL TEM1400 transmission electron microscope equipped with an Olympus SIS Quemesa 11 Mpxl camera. To identify the cell of interest, low magnification (100x) EM views were compared with DIC images of the target cell and the surrounding cells, taken before EM-preparation. Once found, the cell was imaged at low magnification (1000x) and higher magnification (12kx) of the organelles of interest in every section.
Correlation:
Light- and electron microscopic images were correlated by overlaying one Z-plane of the EM serial images with one Z-plane of the fluorescence images. Matching of Z-planes was based on the fluorescent fiducial markers LAMP1-mCherry for lysosomes or MitoTracker for mitochondria. The LM- and EM-images were scaled and aligned using GIMP, and employing the same fiducial markers.

In vitro phosphorylation

GST-PSEN2 constructs were eluted (end-over-end, 10 min, 4°C) with 2X 1 volume elution buffer (3.5 mM reduced glutathione - acros organics; 50mM TrisHCl pH8.0) and 1 volume supplemented elution buffer (3.5 mM reduced glutathione; 150mM NaCl; 5 µM $CaCl_2$ 0.1% β-ME; 50mM TrisHCl pH8.0). Elutions were pooled and dialyzed (4 °C; O/N) against 250 volumes of storage buffer (5% glycerol; 1mM DTT; 20mM TrisHCl pH7.5). Substrates were concentrated (Vivaspin, 10 kDa cutoff, Millipore) and frozen until further use.

Protein kinases used (Aurora A; PKA; Cdk2; CKI; CKII) were expressed and purified in house.

In vitro kinase assays were as follows: bacterially expressed and purified substrate (2 µg, unless stated otherwise) was incubated with excess active kinase for 1 h at 30°C ([γ-^{32}P]-ATP (0.2 µCi; 3000 Ci/mmol; Perkin-Elmer Easytides); 100 µM ATP; 2 mM MgAc; 0.05 mg/ml BSA; 1 mM DTT; 20 mM TrisHCl, pH7.5). Control reactions lacked he purified kinase or substrate. Reactions were halted with 10 mM EDTA (10 min, room temperature) and boiled in Laemmli buffer. Proteins were separated via SDS-PAGE. Phosphorylation and overall protein content was visualized by autoradiography (Typhoon FLA 9500) and blue-silverstain (Candiano et al., 2004), respectively.

Tandem Mass Spectrometry (MS/MS)

Affinity-purified GST-PSEN2 not eluted from the beads was subjected to an in vitro phosphorylation, as described in the corresponding section except that no [γ-^{32}P]-ATP was used, followed by on-bead trypsin digestion. The resulting peptide mixture was desalted by C18 ZipTip pipette tips (Millipore) and loaded on a LC-Q Exactive Orbitrap (Thermo Fisher Scientific) MS system. Data analysis was executed by using the MASCOT (Matrix Science) search engine together with the Proteome Discoverer 1.4 PhosphoRS 3.0 workflow. The phosphorylation site assignment was also manually verified.

Plasma membrane sheets preparation

For plasma membrane sheets from MEF dKO double rescued with GFP-PSEN1 and TagRFP-PSEN2 were prepared as previously described (Chaney and Jacobson, 1983). Briefly, cells were grown to 60-70% confluency on glass coverslips were washed twice with PBS+/+, then washed with coating buffer (CB: 20 mM MES, 135 mM NaCl, 0.5 mM CaCl2, 1 mM MgCl2, pH 5.5) on ice. Cells were incubated subsequently with 1% cationic colloidal silica beads in CB and with 1mg/ml polyacrylamide in CB to coat the apical membranes (each for 1 min). After three washes with CB, cells were incubated with hypotonic buffer (2.5 mM imidazole, pH 7.0, supplemented with EDTA-free PI) for 10 min. To prepare plasma membrane sheets, shear force was applied on the coverslip using a syringe with a blunt tip needle held in a 30° angle (with respect to the coverslip). Plasma sheets were fixed for 15 min in 4% paraformaldehyde at 4°C and mounted on slides with Mowiol.

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Supplemental data

FIGURE S1. Related to Figure 2

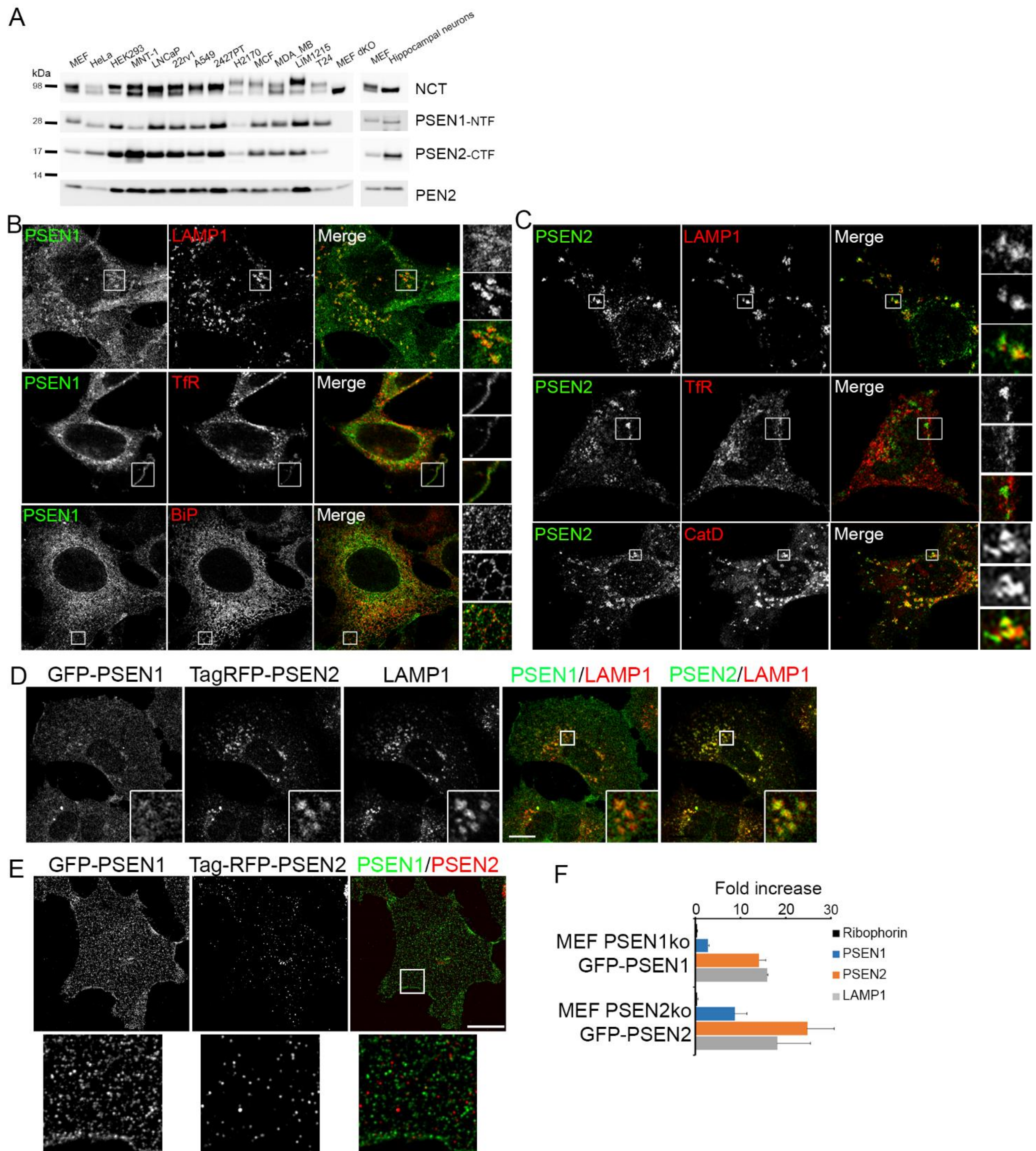

Figure S1: (A) Western blot analysis of total cell lysate (20 µg protein) of different cell lines demonstrates high variability in endogenous PSEN1 and PSEN2 expression. Cancer cell lines derived from in particular melanoma (MNT-1), prostate (LNCaP) and lung carcinoma (22rv1, A549, 2427PT) have concomitant higher expression of PSEN2 and other subunits like mature NCT and PEN2 indicating higher γ-secretase complexes and activities.
(B-D) Differences in subcellular localization are recapitulated in (B) single PSEN1 and (C) single PSEN2 knockout MEF rescued with the respective GFP-tagged PSEN1 and PSEN2, as well as in (D) double knock out rescued with both GFP-PSEN1 and TagRFP-PSEN2. These results underscore that stable low exogenous expression of one PSEN does not influence subcellular distribution of the other. Insets: restricted distribution of GFP-PSEN2 and not GFP-PSEN1 in LAMP1-positive organelles. GFP-PSEN1 co-localizes with the ER-marker BIP in reticular patterns and with the transferrin receptor (TfR) at the cell surface. Bar= 10 µm.
(E) Confocal analysis of isolated plasma membrane sheets prepared from MEF dKO double rescued cells (GFP-PSEN1 and TagRFP-PSEN2) shows that GFP-PSEN1 is more abundant at the cell surface than Tag RFP-PSEN2 complexes confirming cell surface biotinylation data (Figure 1). Lower panel represent a magnified view of the region represented by a square in the merged panel. Note the overall lack of co-localized spots suggesting that the different PSEN complexes exist in distinct microdomains. Bar= 10µm.

(F) Quantitative Western blot analysis of total cell lysate and LE/LYS magnetically isolated from single PSEN1 KO MEFs rescued with GFP-PSEN1 and single PSEN2 KO MEFs rescued with GFP-PSEN2. For both cell lines, endogenous PSEN2 as well as GFP-PSEN2 co-enriches with LAMP1. The ER-marker ribophorin is used as a negative control for the purification of LE/LYS. Data are the average of two experiments.
Related to Figure 1.

FIGURE S2. Related to Figure 3

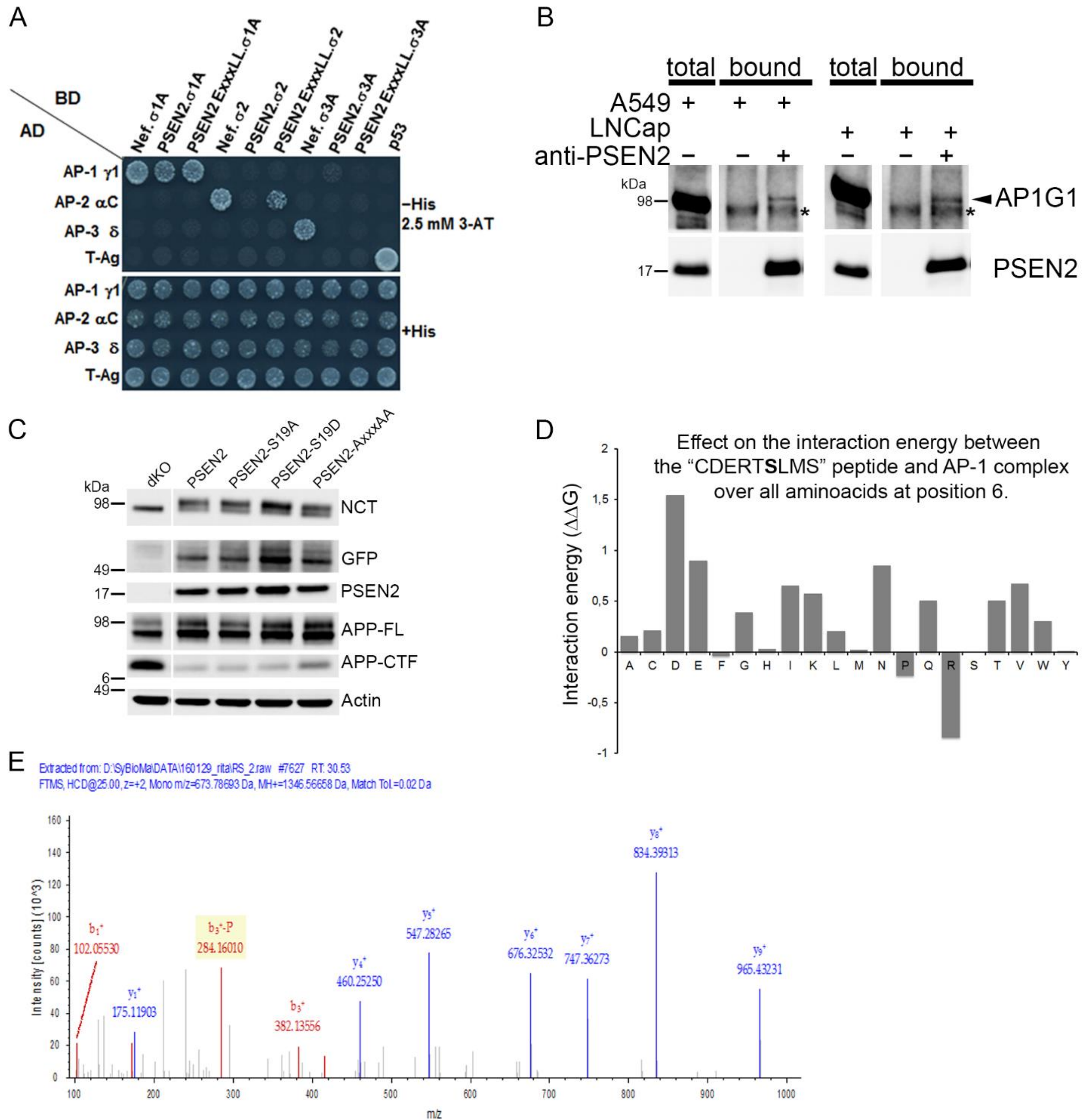

Figure S2:

(A) Y3H analysis shows that substitution of Leu for Met21 allows the PSEN2 tail to interact not only with AP1γ1σ1 but also with AP-2 αCσ2. In none of the occasions, interaction was detected with AP3 hemicomplexes.

(B) Westernblot analysis of co-immunoprecipitations using anti-PSEN2 on CHAPSO extracts (150 µg of total protein) from A549 and LNCaP cells. These cells were chosen because of their high endogenous PSEN2 expression levels. Endogenous AP1G1 (arrowhead) co-immunoprecipitates with PSEN2. * Non-specific band that also is present in control lanes (bound fraction without anti-PSEN2).

(C) Western blot analysis (20 µg total lysate/lane) of dKO MEFs stably rescued with GFP-PSEN2 mutants $S_{19}A$, $S_{19}A$, and AxxxAA with the indicated antibodies. All three PSEN2 mutants fully rescue NCT maturation and APP processing indicating restoration of γ-secretase activity.

(D) Graphical representation of the predicted effect on the interaction energy (ΔΔG) between the binding motif "CD**ERTSLM**S" and the AP-1 γ1σ1 hemicomplex while changing the Ser19 to all other amino acids. Note that next to Asp (D) also the phosphomimic Glu (E) as well as the structurally related Asn (N) give the highest positive ΔΔG-values indicating that they are predicted to disfavor interaction.

(E) MS/MS spectrum of m/z 673,79 corresponding to mono-phosphorylated TSLMSAESPTSR after phosphorylation of GST-$N_{1\text{-}86}$PSEN2 with Aurora kinase. The TSLMSAESPTSR peptide is shown to be phosphorylated (on S19 (as confirmed by a PhosphoRS 3.0 Site Probability of 99.7% and also by manual verification). Together with m/z 681,78 (RT 27,18 min), the oxidized methionine counterpart of this peptide, they are the only phosphorylated forms of the TSLMSAESPTSR peptide in this condition.

Related to Figure 3.

FIGURE S3. Related to Figure 4

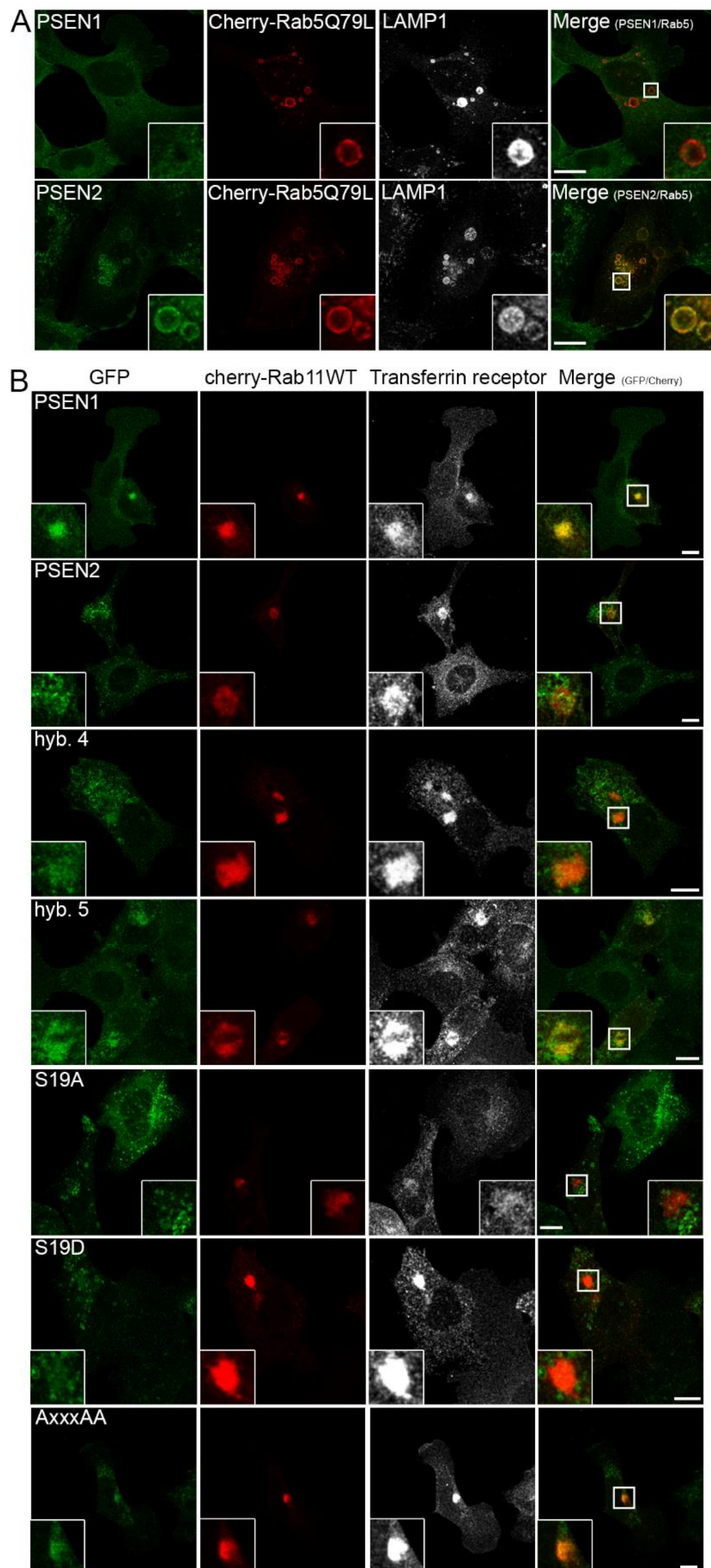

Figure S3: (A) MEF dKO rescued with either GFP-PSEN1 or GFP-PSEN2 were transiently transfected with Cherry-Rab5Q79L to block exit of endosomal cargo from early endosomes, fixed and processed for confocal microscopy. GFP-PSEN2 but not –PSEN1 co-accumulates with LAMP1 (white) in enlarged endosomes (merged inset) suggesting that GFP-PSEN2 is delivered to this early endosomal sorting compartment. Bar: 10 µm.
(B) MEF dKO rescued with GFP-PSEN1, -PSEN2, hybrid PSEN proteins (hyb4 and hyb5, see Figure 2A) and –PSEN2 transport mutants (S_{19}A, S_{19}D and AxxxAA, see Figure 3A) were transiently transfected with Cherry-Rab11-wild type. Transient high overexpression of wild-type Rab11 causes the jamming of cargo like transferrin receptor (TFR). Co-

accumulation of GFP-PSEN1 indicates that PSEN1 traffics through or via the Rab11-recycling compartment as opposed to GFP-PSEN2 which does not accumulate. Replacing the N-terminus of PSEN1 with the equivalent of PSEN2 (hyb4) results in a loss of Rab11 co-localization while reversely exchanging the N-termini of PSEN2 with the one of PSEN1 (hyb5) results in co-accumulation. The $S_{19}A$ and $S_{19}D$ mutants of PSEN2 also fail to co-localize with exogenous Rab11. Instead, the AxxxAA mutant, which abrogates interaction with AP-1, now readily co-accumulates with the TFR in the Rab11 recycling compartment. This suggests that the AP-1 interaction prevents PSEN2 from being sorted via the Rab11 recycling compartment. Bar: 10 μm.
Related to Figure 4.

FIGURE S4. Related to Figure 5

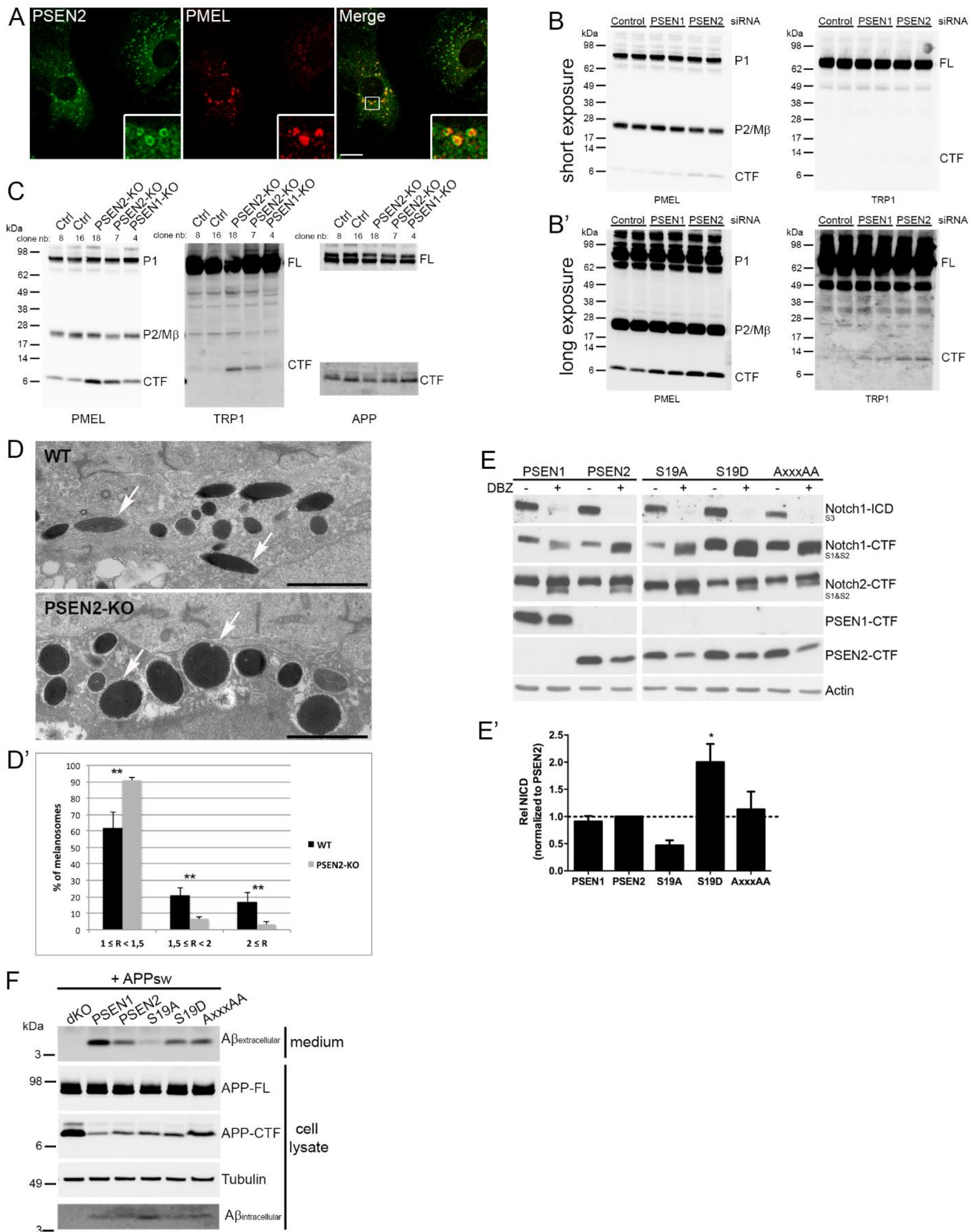

Figure S4: (A) Confocal analysis of MEF dKO rescued with GFP-PSEN2 (green) transiently expressing PMEL (red). Inset shows the localization of exogenous PMEL in GFP-PSEN2 positive endosomes. Bar=10μm. (B, B') MNT-1 cells transfected with Ctrl, PSEN1 or PSEN2 siRNA were analyzed 48 h later by western blot. Antibodies against the CTF part of PMEL and TRP1 were used to detect full-length and processed fragments. (B') is a long exposure of (B). PMEL-CTF and TRP1-CTF are the only fragments which accumulate upon downregulation of PSEN2. (C) PSEN1 and PSEN2 knockout MNT-1 cells were analyzed by Western blot. Antibodies against the CTF of PMEL, TRP1, and APP were used to detect full-length and processed fragments as indicated. P1: immature core-glycosylated PMEL form, Mβ: product of proprotein convertase cleavage; CTF: CTF fragment; FL: full-length. (D) EM analysis of epon-embedded RPE of WT and PSEN2-KO mice. Note the presence of normal ellipsoidal-shaped melanosomes in WT mice versus

abnormal big round-shape melanosomes in PSEN2-KO mice (white arrows). Bar= 2 μm. (D') Ratio (marked as "R") between maximum width and length of an average of 150 melanosomes per condition. Melanosomes are significantly less elongated in PSEN2-ko RPE (**P<0.01).

(E) Delta ligand-induced processing of endogenous Notch1 in PSEN dKO MEFs rescued with GFP-PSEN1, -PSEN2 and transport mutants $S_{19}A$, $S_{19}D$ and AxxxAA (see Figure 3A). Westernblot analysis of the levels of Notch1- and Notch2-CTF and NICD production in the absence (-) and presence (+) of a γ-secretase inhibitor,DBZ (Dibenzazepine, 0.2 μM, 16 h). Note clear accumulation of the respective CTF fragments of endogenous Notch1 and Notch2 which are the substrates of γ-secretase. The cleaved NICD1 specific antibody (Val1744) only recognizes γ-secretase processed Notch1 but not Notch2. (E') Quantification of NICD production of Western blots in (E). No differences were found between PSEN1 and PSEN2, but the PSEN2-$S_{19}A$ decreased NICD production, while significantly more NICD is produced in the case of PSEN2-$S_{19}D$ expression (Mean ± SEM, n=3).

(F) MEF dKO and rescued cell lines were transiently transfected with APPswedish (APPsw). After 24h, conditioned media were collected and cells were lysed. Total lysates (10 μg) were analyzed by Western blot to measure intracellular Aβ and transfection efficiency, and conditioned medium (25 μl) to evaluate secreted/extracellular Aβ levels.
Related to Figure 5.

FIGURE S5. Related to Figure 6

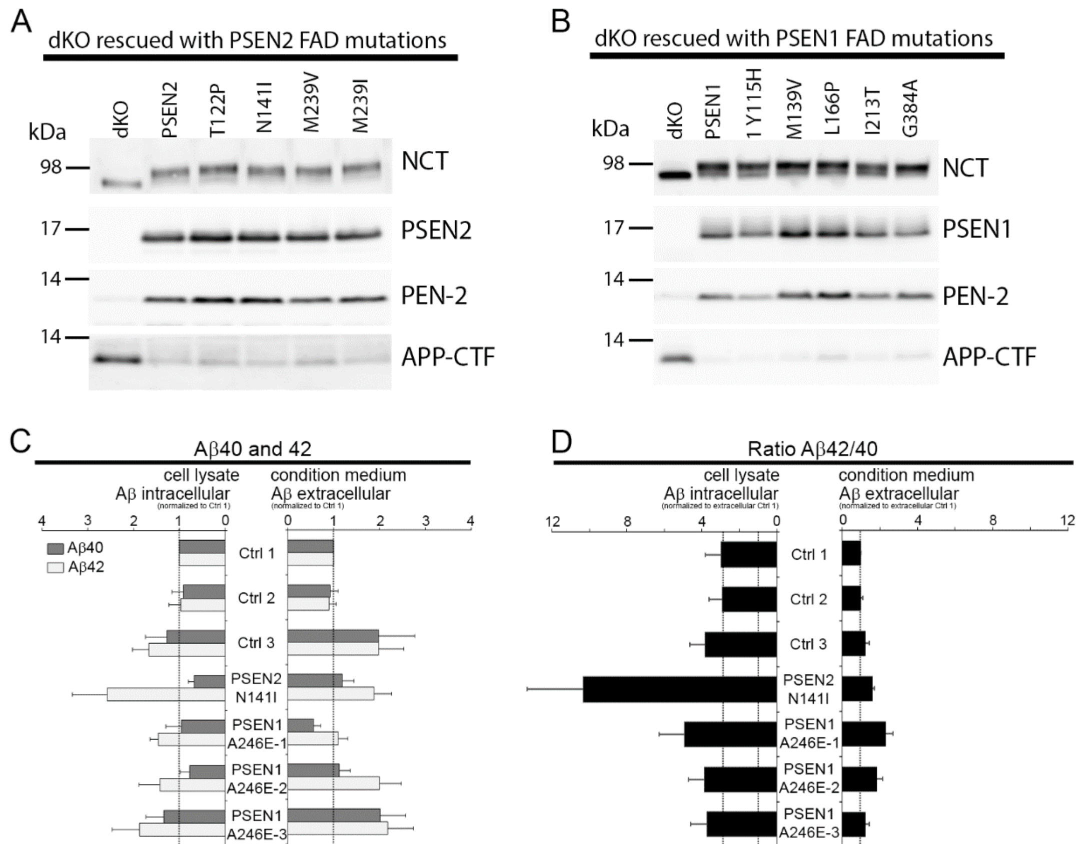

Figure S5:
(A and B) Western blot analysis of dKO MEFs stably rescued with (A) PSEN2-FAD mutants (T122P, N141I, M239V, M239I), and (B) PSEN1-FAD mutants (Y115H, M139V, L166P, I213T, G384A) displaying restored NCT maturation, PEN2 stabilization and lack of APP-CTF accumulation underscoring rescued and normalized γ-secretase complex formation and activity.
(C and D) Quantification by ELISA of intracellular and extracellular Aβ40 and Aβ42 levels in the indicated patient primary fibroblast cells transiently transfected with C99-3XFLAG. For all quantification, Aβ signals were corrected for the level of C99-3XFLAG expression, and normalized to control 1 (mean ± SEM, n=6).
Note the selective strong increase of intracellular Aβ42 and the Aβ42/40 ratio in the FAD PSEN2-N141I cell line. Related to Figure 6.

Legends to supplemental Movies: Related to Figure 4

Supplemental Movie S1:

SIM-CLEM of GFP-PSEN2-S_{19}A-rescued dKO MEFs transiently overexpressing LAMP1-mCherry.

A SIM image (x-y resolution ~125 nm; z-resolution ~300 nm) was superimposed on a stack of three consecutive EM transmission sections of 50 nm (total 150 nm). mCherry fluorescence was used as fiducial label to optimally position the SIM image on the organelles identified ultrastructurally as LE/LYS. The GFP-PSEN2-S_{19}A fluorescence is clearly localized in tubules and clathrin-coated vesicles in close proximity to TGN stacks. One of the frames of this movie was used in Figure 4G').

Supplemental Movie S2:

SIM-CLEM of GFP-PSEN2-S_{19}A-rescued dKO MEFs stained with Mitotracker. A SIM-image was superimposed on a stack of eight consecutive EM transmission sections of 50 nm (total 400 nm). Mitotracker was used as fiducial marker to align the SIM image with the mitochondria ultrastructure. The GFP-PSEN2-S_{19}A fluorescence is concentrated in a rim neighboring several Golgi stacks. The presence of clathrin-coated vesicles and dilated tubules identifies this region as TGN.