Supplementary information

Alzheimer's disease mutations in APP but not γ-secretase modulators affect epsilon-cleavage-dependent AICD production

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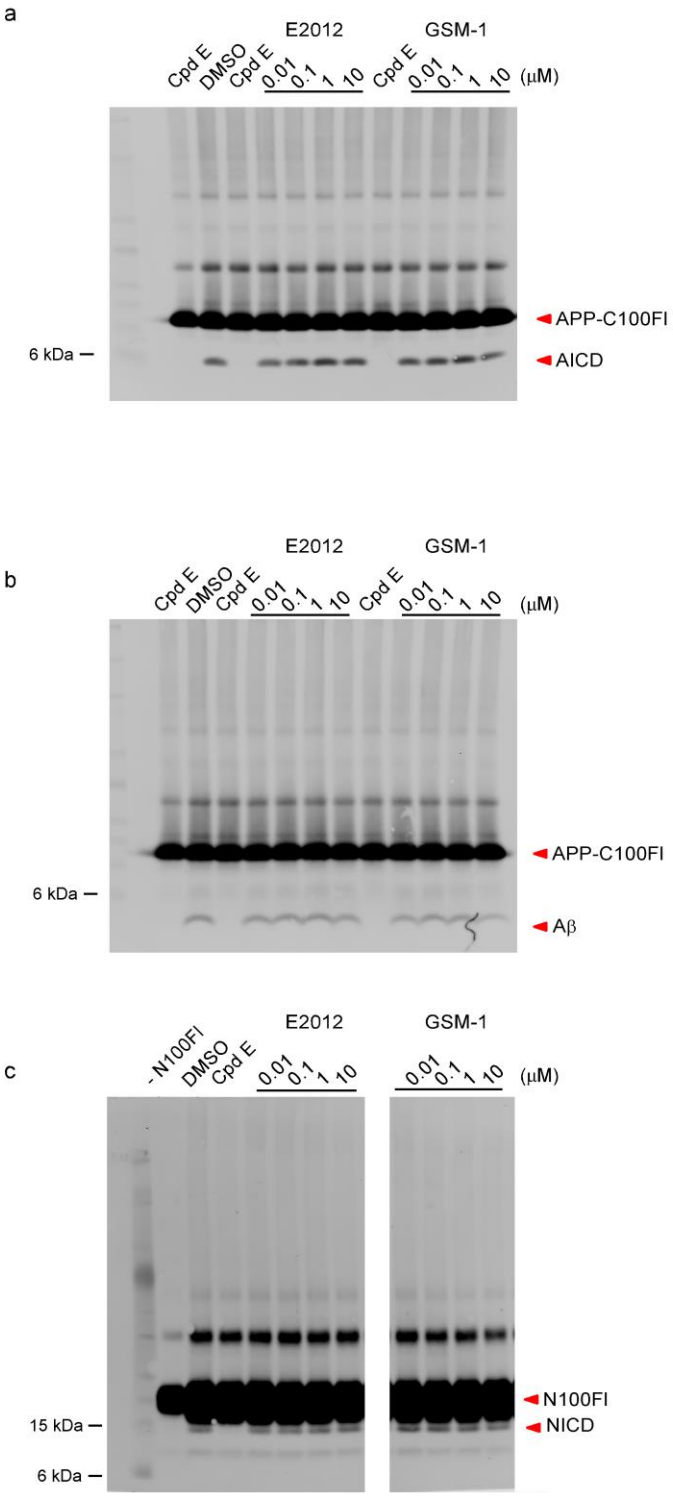
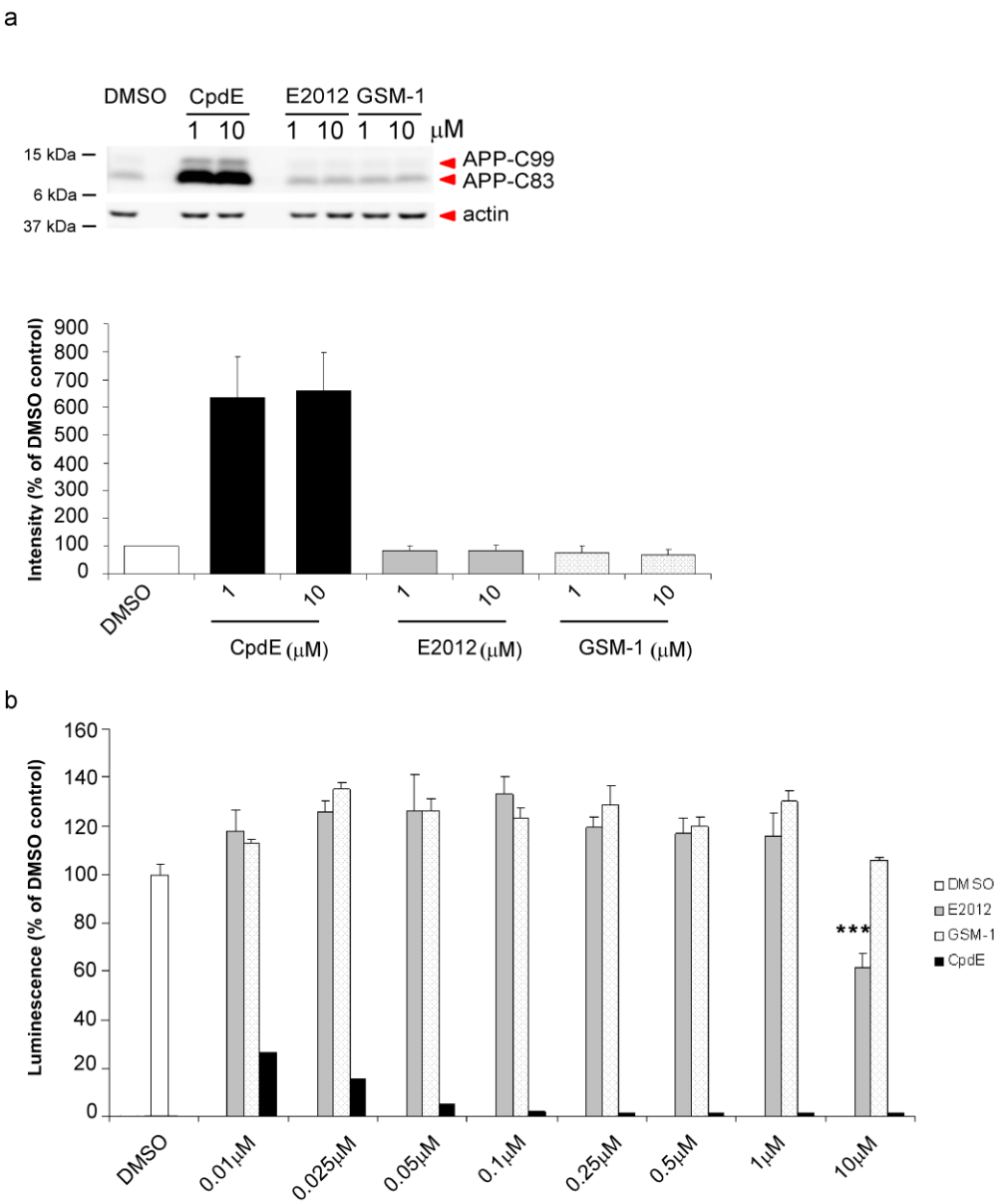
Supplementary Figure S1



Corresponding full Western Blots from Figure 2. a) AICD, b) Aβ and c) NICD

Supplementary Figure S2

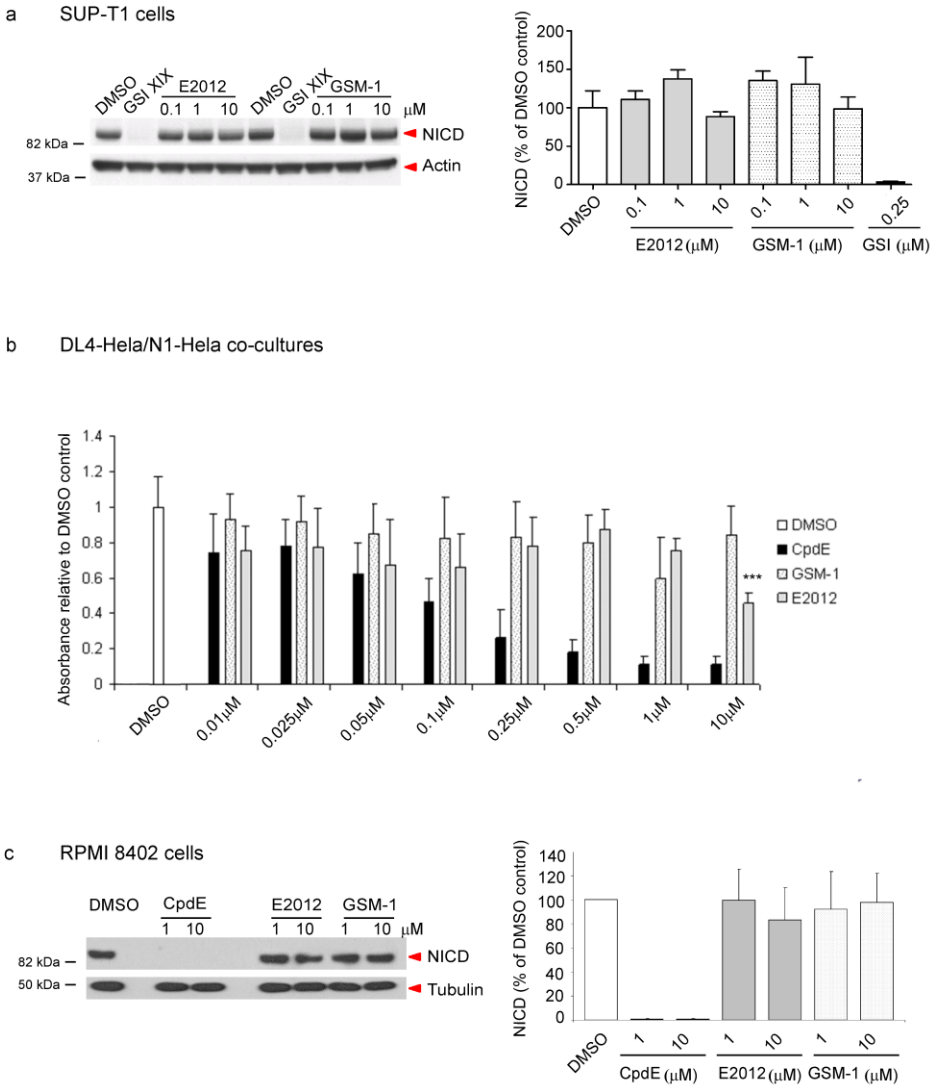


GSMs do not modulate ε-site cleavage of APP in cell-based assays at concentrations up to 10-fold cellular IC_{50} . a) γ-Secretase-dependent APP C-terminal fragments, APP-C99 and APP-C83, in GSM-treated HEK293T overexpressing APP with the Swedish KM670/671NL mutation. Following treatment with 1 µM or 10 µM of GSMs cells were harvested and total proteins were extracted with 1 % NP40. After normalization, proteins were loaded on a SDS gel and APP-CTFs

detected by WB using an antibody against the C-terminal region of APP (upper panel). Actin served as a loading control. Densitometric analysis (lower panel) by *ImageJ* software demonstrated the accumulation of APP-C99 and APP-C83 after incubation with 1 μM or 10 μM of Compound E (CpdE), a potent γ-secretase inhibitor. In contrast, in the same range of concentrations, GSMs did not induce APP-CTF accumulation. A representative Western blot of two independent experiments with similar results is shown. Data from densitometric analyses are plotted as means ± SD.

b) Effect of a GSI and GSMs on γ-secretase-dependent cellular AICD production. The luminescence emitted by T-20 cells directly reflects AICD-Gal4VP16 formation[43]. γ-Secretase inhibitor Compound E potently inhibits the luminescence signal in a dose-dependent manner. In contrast, up to 1 μM GSMs has no effect while 10 μM E2012 (but not GSM-1) lowered AICD production by ~40% in this specific assay. Results are expressed as mean ± SD (n = 3). Statistical analyses were performed using a two-tailed Student's t-test with ***$p < 0.001$ versus DMSO control.

Supplementary Figure S3



GSMs do not modulate ε-site cleavage of the Notch-1 receptor in cell-based assays at concentrations up to 10-fold cellular IC_{50}. a) GSMs have no effect on NICD production in SUP-T1 cells treated with 0.1, 1 and 10 μM of either E2012 or GSM-1. GSI XIX (0.25 μM) was used as a positive control for the inhibition of the γ-secretase-dependent Notch processing. Actin serves as an equal loading control. Densitometric analysis of the NICD bands indicates that, in contrast to GSI XIX, NICD formation is not reduced by any of the GSMs. b) GSMs do not modulate ε-site Notch products in a luciferase-based Notch-specific cellular system. A co-culture

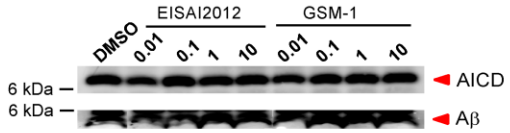
of HeLa cells overexpressing either the ligand Delta4 (DL4-Hela) or the Notch-1 receptor (N1-Hela) was treated with the indicated concentrations of CpdE, GSM-1 or E2012. Neither GSM-1 nor E2012 modulated significantly NICD formation at concentrations up to 1 μM. As expected, CpdE inhibited Notch processing in a dose-dependent manner, with an apparent IC_{50} of 100 nM. At 10 μM E2012 (but not GSM-1) reduced the intensity of the luminescence by ~50 %. c) GSMs do not inhibit NICD formation in RPMI8402 cells treated with 1 or 10 μM of GSMs or CpdE for 24 hours prior to total protein extraction.

a, c: Representative Western blots of two independent experiments with similar results are shown (left). Data from densitometric analyses of two independent experiments are plotted as means ± SD (right).

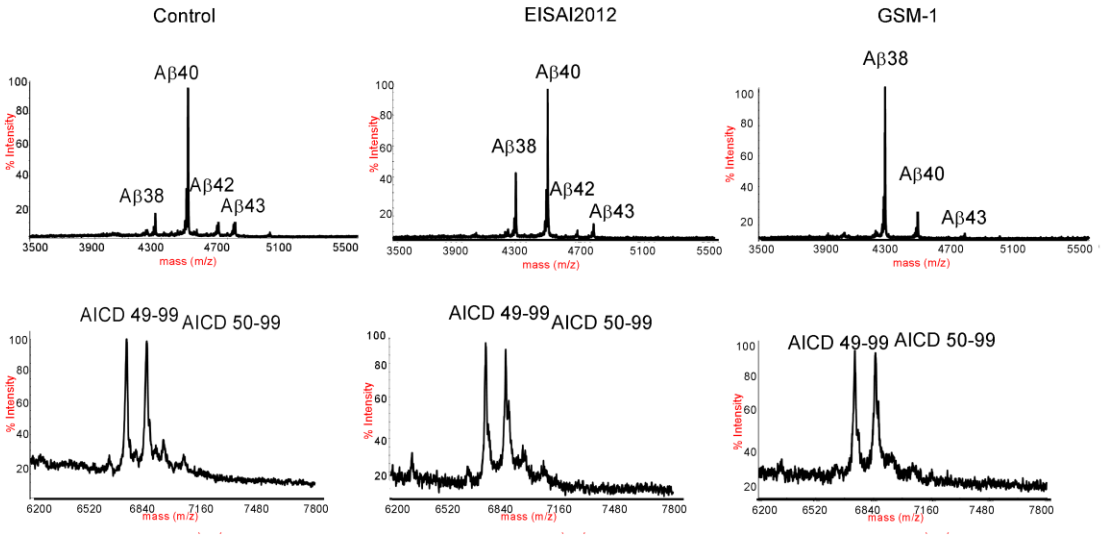
b: Data are expressed as mean ± SD ($n \geq 7$). Statistical analyses were performed using a two-tailed Student's t-test with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus DMSO control.

Supplementary Figure S4

a

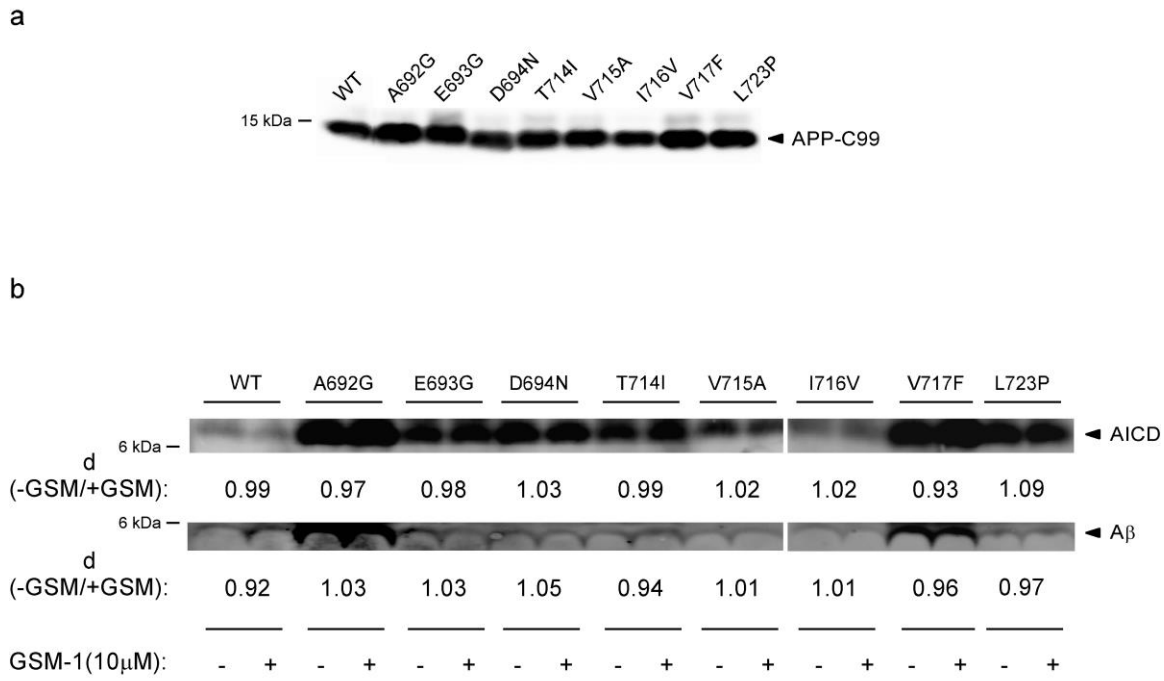


b



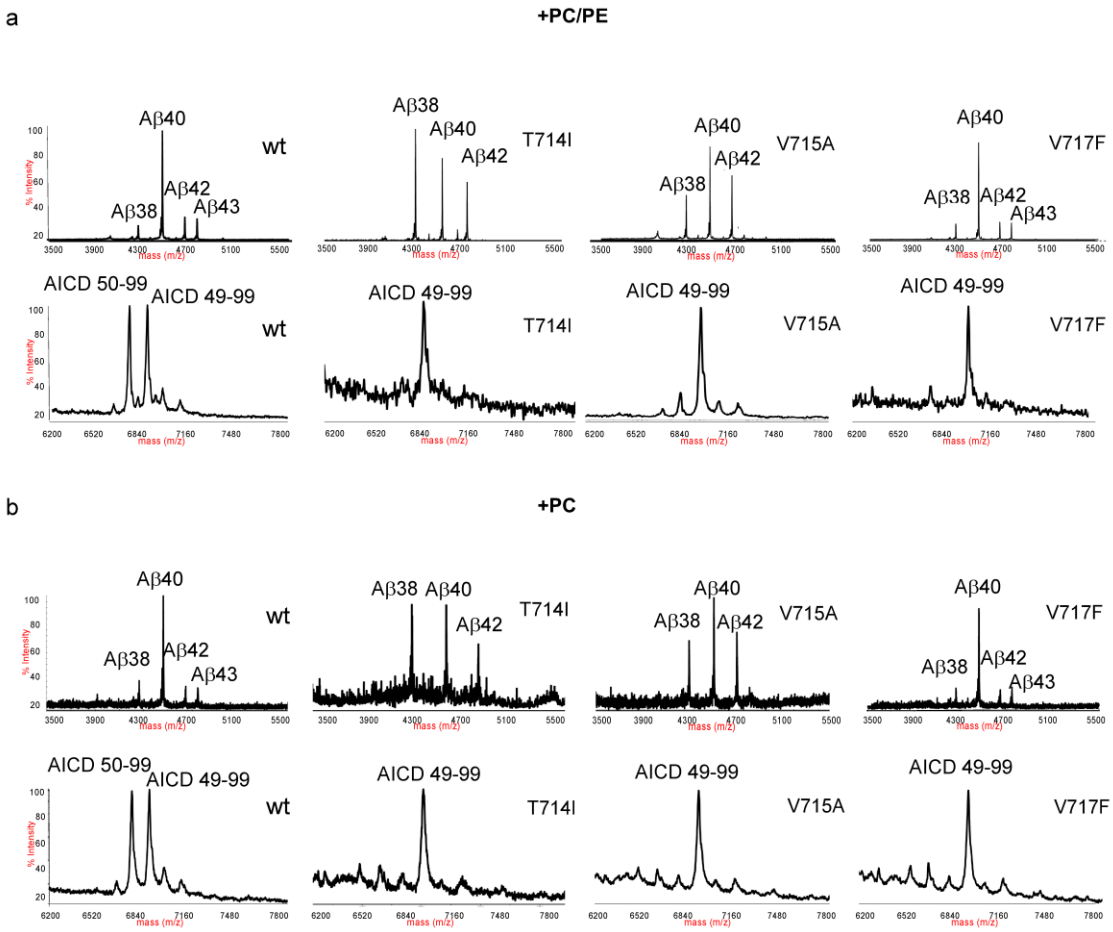
GSMs alter γ-site processing, but do not modulate ε-site processing of His6-tagged wild-type APP-C99 by purified γ-secretase. a) WB analysis of Aβ and AICD cleavage products from γ-secretase activity assays in the presence of different concentrations of GSMs ranging from low nanomolar to low micromolar. b) Corresponding Aβ and AICD profiles determined by IP/MS. MALDI-TOF spectra demonstrate the potency of GSMs on the γ-site, as witnessed by lowered Aβ42 production associated with a shift of Aβ production towards shorter Aβ38. Note that results obtained with the His6-tagged APP-C99 substrate are highly similar to those obtained with the Flag-tagged APP-C99 substrate (Fig. 2), demonstrating that these different tags do not alter the effect of GSMs on both γ- and ε-sites.

Supplementary Figure S5



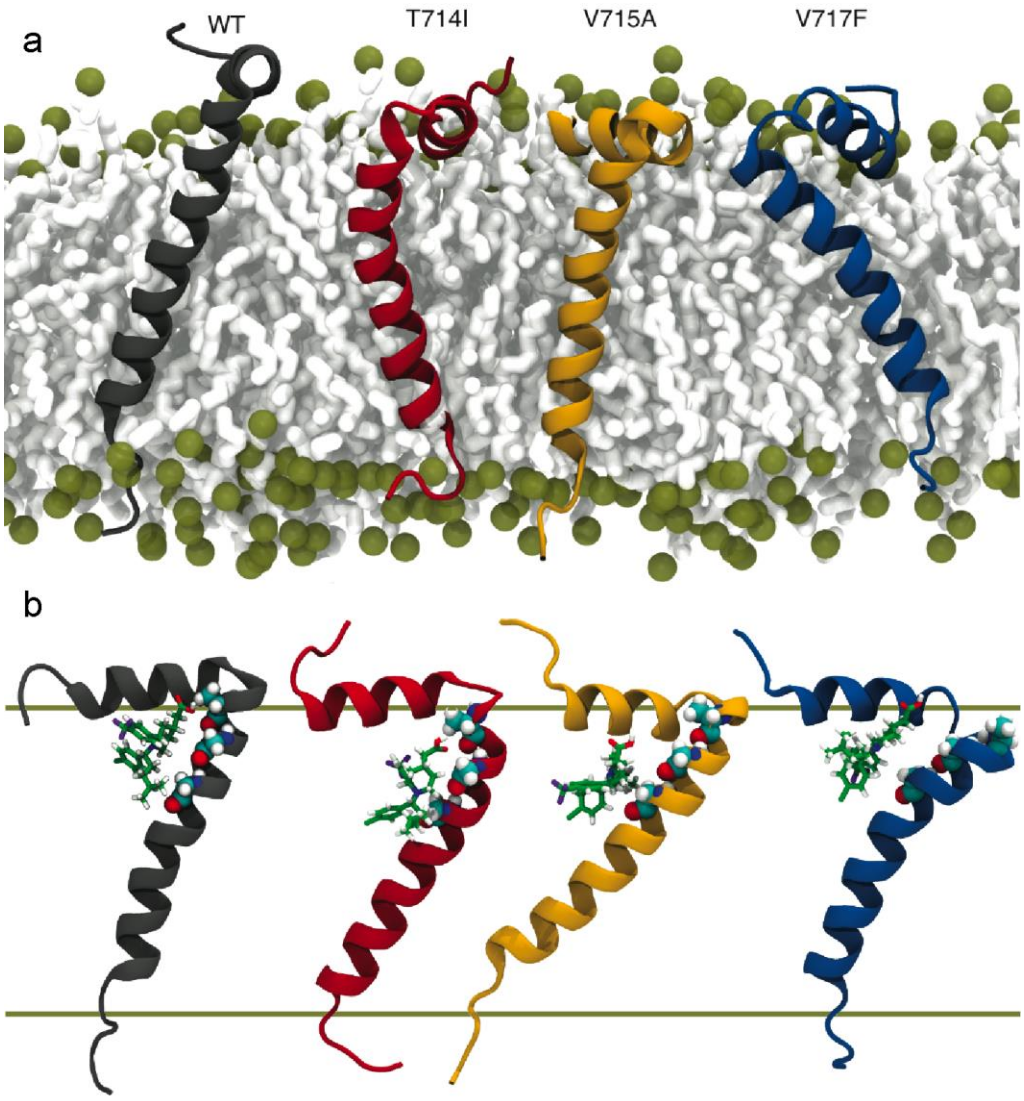
Processing by purified γ-secretase of His6-tagged APP-C99 FAD mutants in the presence or absence of GSM-1. a) His6-tagged APP-C99 recombinant substrates with FAD mutations were produced in *E.coli* and purified on Nickel-bound agarose beads. After elution, proteins were quantified by BCA assay and normalized. Equal amounts of proteins were then verified by WB using a C-terminal anti-APP antibody. b) Wild-type and FAD mutant APP-C99 substrates were used for activity assays with purified γ-secretase in the presence (+) or absence (-) of 10μM GSM-1. After 4 h at 37 °C the enzymatic reactions were probed by WB for AICD or Aβ products. All APP-C99 substrates with FAD mutations were processed by the γ-secretase complex. as estimated by densitometric analysis (d), 10 μM of GSM-1 did not affect the production of both γ- and ε-site-dependent Aβ and AICD. d: ratio between non-treated (-) and GSM-1 treated (+) AICD (upper panel) and Aβ (lower panel) cleavage products.

Supplementary Figure S6



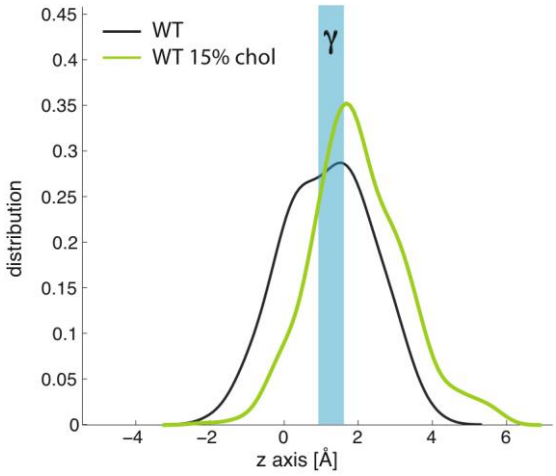
Processing of His6-tagged WT or FAD APP-C99 substrates by purified γ-secretase reconstituted in phosphatidylcholine in combination with phosphatidylethanolamine (+PC/PE), or in phosphatidylcholine alone (+ PC). IP/MS profiles of Aβ and AICDs generated in the presence of a) 0.1% PC and 0.025% PE (+PC/PE) or b) 0.1% PC alone (+PC). Note that Aβ and AICDs profiles are highly similar, demonstrating that the addition of PE does not affect γ- or ε-sites.

Supplementary Figure S7



Molecular docking and MD simulations of GSM-1 bound to APP-C99. a) Different orientations in the membrane of APP WT and mutants TMD are reported with respect to Figure 5 in the main text. b) GSM-1 binding to WT, T714I, V715A and V717F APP obtained using SWISSDock and BSP-SLIM revealed that the compound binds to the $G_{700}XXXG_{704}XXXG_{708}$ motif (in ball representation) with a conserved and stable conformation. The wild-type system is equilibrated using MD simulation and confirmed a stable pose of GSM-1 compound in dynamics.

Supplementary Figure S8



Spatial distribution of the Aβ42 cleavage site. The position for the APP wt inserted into a pure POPC membrane and a POPC membrane containing 15% of cholesterol are shown in black and green, respectively.

Supplementary methods

Cell culture.

Human embryonic kidney cells (HEK293T) were routinely grown in DMEM with 10 % fetal bovine serum (FBS) and penicillin/streptomycin in a humidified 5 % CO_2 atmosphere. The HEK-293 cell line expressing stably APP with double Swedish mutation (HEK APPSwe) or RPMI8402[44] were maintained in DMEM, 10 % FBS supplemented with 300 µg/ml Geneticin G418 or zeocin 250 µg/ml, respectively (Invitrogen Carlsbad, CA, USA). T-Rex HeLa cells stably expressing the repressor of tetracyclin (Invitrogen Carlsbad, CA, USA) and APP (T20 cell line)[43] were maintained in Eagle's Minimum Essential Medium with 10 % FBS, 2 mm L-glutamine, 1 % penicillin/streptomycin, and 5 µg/ml blasticidin. Cells were plated in 96-well-plates, and incubated for 24 h with tetracycline and GSMs/GSIs. SUP-T1 cells were grown as previously described[45]. The luminescence signals were measured with a Tecan Infinite plate reader, using the Bright-glo luciferase kit (Promega, Dübendorf, Switzerland). For the Notch-based coculture assay, HeLa cells overexpressing either the ligand Delta4 (DL4-Hela) or the Notch-1 receptor (N1-Hela) were mixed in a 1:1 ratio (5000: 5000 cells/well) and added to 384 well plates using multidropCombi plate dispenser. Luciferase readout was measured using dual luciferase assay system.

Statistical analysis.

Results are expressed as mean ± SD of $n \geq 3$ independent samples. All statistical analyses were performed using a two-tailed Student's t-test. $p < 0.05$ was considered significantly different from control.

Supplementary References:

43. **Liao, Y.F., Wang, B.J., Cheng, H.T., Kuo, L.H. & Wolfe, M.S. Tumor necrosis factor-alpha, interleukin-1beta, and interferon-gamma stimulate gamma-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway. *J Biol Chem* 279, 49523-32 (2004).**

44. **Weng, A.P. et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306, 269-71 (2004).**

45. **Jeffries, S., Robbins, D.J. & Capobianco, A.J. Characterization of a high-molecular-weight Notch complex in the nucleus of Notch(ic)-transformed RKE cells and in a human T-cell leukemia cell line. *Mol Cell Biol* 22, 3927-41 (2002).**