

Figure S1: C83 cleavage by γ-secretase (A) Relative reporter gene activity using C83-T4LrTA as substrate. Rho(4M)-TEV-site-rTA and Arr(3A)-TEV serve as positive control. (B) Reporter gene activity is abolished in cells with a chromosomal deletion of *PS1* and *PS2*. Activity can be restored to a similar level as the positive Rho-Arr control by transfecting wild type *PS1* (first lane).



Figure S2: Western blot validation of overexpression of γ-secretase. Lane 1: Extracts from cells co-transfected with the four subunits of γ-secretase; Lane 2: Extracts from cells transfected with PS1 wild type; Lane 3: Extracts from non-transfected cells (endogenous control). In HTL WT cells, γ-secretase protein levels were determined by immunoblotting using Anti-Aph1a antibody (Abcam ab12104) (a), Anti-PEN2 antibody (Abcam ab154830) (b), Anti-Nicastrin antibody (Abcam ab122969) (c), and Anti-PS1 CTF (Cell Signaling Technologies 3622S) (d). In HTL PS deletion cells, PS1 protein levels were determined by immunoblotting using Anti-PS1 CTF (Cell Signaling Technologies 3622S) (e). β-actin antibody (Abcam Ab6276) has been used for normalization (lower panel). [&]APH1a isoform. *Antibody cross-reactive bands.



Figure S3: γ -secretase inhibitors do not affect reporter activity in an unrelated Tango assay. All seven inhibitors (100 nM) used in the γ -secretase epsilon-cleavage assay (Figure 1c) have only minor effects on reporter gene activity in the control Rho-Arr Tango assay.



Figure S4: An optimized system for targeted gene deletion using CRISPR/Cas9. (A) Schematic diagram depicting the locations of single guide RNAs targeting human PS gene loci. Guide RNAs were designed using the online CRISPR Design Tool (http://crispr.mit.edu/) and inserted into the modified pX458 vector. Cas9 (light orange shape) guided by sgRNA, which consists of a 20-nt guide sequence (blue) and a scaffold sequence (red), cleaves genomic DNA three base pairs upstream of the requisite 5'-NGG protospacer adjacent motif (PAM (purple); cleavage site indicated by red arrows). The modified pX458 vector is designed to produce one pair of sgRNAs for each gene. (B) Flowchart of targeted gene deletion using CRISPR/Cas9.



Figure S5: Validation of the PS1/PS2 deletion. (A) Relative expression of PS1 and PS2 mRNA. Quantitate RT-PCR using p_{indel} and P_2 primers (Table S1) and GAPDH as an internal reference (error bars=SEM, n=3). (B) PS1/PS2 cDNA PCR validation (P_1 and P_2 as PCR primers). The fragment for the PS1 deletion has a size of 144 bps while the size of the corresponding WT fragment is 289 bps; the fragment for the PS2 deletion has a size of 134 bps while the one of the corresponding WT fragment is 318 bps. (C) PS1/PS2 cDNA sequencing validation (using P_2 as sequencing primer).



Figure S6: Calibration of Aβ peptide levels. Aβ peptide calibration was carried out according to the PerkinElmer AlphaLISA standard protocol. The sensitivity of the Aβ40 kit (AL202 C/F) is 88 pg/mL and Aβ42 (AL203 C) is 300 pg/mL.



Figure S7: A β peptide levels in cell lysate. AlphaLISA assay of lysates from cells transformed with the mutant C99 constructs corresponding to Figure 3. The cell lysate A β 40 (a) or A β 42 (b) peptide levels are close to background (control lane). Error bars=SEM, n=3.

	10	20	30	40	50	60
		.	$\cdots \cdots $		· · · · I · · <u>· ·</u> ·	.
APP	~~~~~LVFFAED~~	~~~~ VGSNK ~	~~~ <mark>GAIIGLM</mark> V	GGVVIATVI	VITLVMI K	~ KK QYTSIHH~
APLP2	~~~~ ESVGPLRED ~~	~~~~ FSLSS ~	~~~ <mark>SALIGLL</mark> V	IAVAIATVI	/ISLVMIR	~ KR QYGTIS H ~
CD44	~~~~SQEGGANTT~~	SGPI R TPQIP	E~~WLIILAS	LLALALILA	/CIAVNSR	~ RR CGQ KKKL ~
CXL16	~~~~GENQKQPEKNA	GPTARTSAT~	·~~~VPVLCL	LAIIFILTA	ALSYVLCK	~ RRR GQS P QS~
DLL3	~~~~ ASALPAAPPGL	RPGDPQR~~~	~~YLLPPALGL	LVAAGVAGA	ALLLVHVR	~ RRGHSQDA ~~
DLL4	~~~~YGFVGSRCEFPV	GLPPSFP~~~	~~~WVAVSLGV	GLAVLLVLL	MVAVAV R	LRLRRPDD ~~
EPCAM	~~~~QTLIYYVDEKAP	EFSMQGLK~~	~~~ A GVIAVIV	VVVIAVVAG	(VVLVIS <mark>R</mark>	~KKRMAKYEK~
EPHA4	~~~~~LEVTTNTVPS	RIIGDGAN~~	~STVLLVSVS	GSVVLVVIL	[AAFVIS <mark>R</mark>	~RRSKYSKAK~
EPHB2	~~~~ FQTMTEAE YQ	TSIQ <mark>EK</mark> LP~~	~~~LIIGSSAA	GLVFLIAVV	VIAIVCNR	~ <mark>RGFERADSE</mark> ~
ERBB4	~~~~DCIYYPWTGHST	LPQHARTP~~	~~~LIAAGVIG	GLFILVIVG	LTFAVYVR	~RKSIKKKRA~
SORC1	LVHAAHLTAAPLVD~~	~~~LTPTH~~	·~~~ <mark>SGSAMLM</mark>	LLSVVFVG~1	LAVFVIY <mark>K</mark> I	FKRRVALPS~~
HLA-A2	~~~GLPKPLTLRWEPS	SQPT~~~~I	PIVGIIAGLV	LFGAVITGA	VAAVMWR	~ RK SS DRK GG~
IFNAR2	~~PLKCTLLPPGQESE	SAESAK~~~~	·~~~ IGGII	TVFLIALVL	[STIVTIK	WIGYICLRN~~
IGF1R	~~~WTDPVFFYVQAKT	GYENFIH~~~	~~~LIIALPVA	VLLIVGGLV	[MLYVFHR	~RNNSRLGNG~
LRP1B	~~~~~QCERPAPKSS	KSDHISTR~~	~~ <mark>SIAIIVPLV</mark>	LLVTLITTL	IGLVLCK	~RKRRTKTIR~
TIE1	~~~~GLQAEGPVQESR	AAEEGLDQQ~	LILAVVGSVS	ATCLTILAA	LTLVCIR	~RSCLHRRRT~
Notch1	~~~~~VQSETVEPP	PPAQL <mark>H</mark> ~~~~	·~~~FMYVAA	AAFVLLFFV	GVLLS	~ KRRRQH GQ L ~
Notch2	~~~~~ VVSE SL	TPERTQ~~~~	~~~LLYLLAV	AVVIILFII	LGVIMAK	~RKRKHGSLW~
Notch3	~~~~ VR GEPL	EPPEPS~~~~	VPLLPLLVA~	GAVLLLVIL	/LGVMVAR	~RKREHSTLW~
CDH2	~~~~~SNGDCTDVD	RIVGAGLGTG	A~~~IIAILL	CIIILLILVI	MFVVWMK	~RRDKERQAK~
DCC	~~~~PPIGQMHPPHGS	VTPQKNSN~~	~LLVIIVV _T V	GVITVLVVV	IVAVICIR	~RSSAQQRKK~
DLL1	~~~~GPAVVDLTEKL	EGQGGPFP~~	WVAVCAGVI	LVLMLLLGC2	AVVVCVR	LRLQKHRPP~~
Ephrin-B1	~~SGPGASGGSSGDPD	GFFNSK~~~~	VALFAAVGAG	CVIFLLIII	?LTVLLI K	LRKRHRKHT~~
Ephrin-B2	~~~~GNSAGHSGNNI	LGS <mark>E</mark> ~~~~V	ALFAGIAS~G	CIIFIVIII	LVVLLIK	YRRRHRKHS~~
JAG2	~~~LLLAVTEVKVETV	VTGGSST~~~	GLLVPVLCGA	FSVLWLACV	/LCVWWIR	~KRRKERERS~
ILIRI	~~~~THGIDAAY	IQLIYPVTNF	'QKH~~~~MIG	ICVTLTVII	CSVFIYK.	IFKIDIVLW~~
LRP1	~~~PPHMTGPRCEEHV	FSQQQPGH~~	~~~IASILIPL	LLLLLVLVI	AGVVFWYK	~RRVQGAKGF~
MUCI	~VSVSDVPFPFSAQSG	AGVPG~~~WG	TALLVLVCVL	VALAIVYLIA	ALAVCQCR	~RKNYGQLD1~
NRG1	~~~ENVPMKVQNQEKA	EELYQKR~~~	~~~VLTITGI	CIALLVVGI	ICVVAYOR	I'KKQRKKLH~~
NRG2	~~~~PLRLYMPDPK	QKAEELYQKR	~~~VLTITGI	CVALLVVGI	CVVAYCK	I'KKQRKQMH~~
Nectini	~~~~FPYTPSPPEHG	RRAGPVPT~~	~~ALIGGVAG	SILLVLIVV	GIVVALR	~RRRHTFKGD~
PCDHGC3	~~RAEFPSGSAPREQK	KNLTFY~~~~	·····	LVSVGFVVT	7FGVIIFK	VYKWKQSR~~~
SORTI	~LKKKCTSNFLSPEKQ	NSKSN~~~~~	SVPIILA	TVGLMLVTV	AGVLIVK	~KYVCGGRFL~
NOTCH4	~~~~~VHPHAG~TA	PPANQLP~~~	~WPVLCSPVA	GVILLALGAI		
SCNZB	~~~GRIHLQVLMEEPP	ERDST~~~~~		SVGGFLAVV.		CVRRKKEQK~~
SURCS	EVI YVIII DOMOOF	SSAGESSS~~				
GHK DUUN1	~~~EVLIVILPOMSOF	DVERGU		CVA CWI AT CO	JEVELESK,	QCKIKMLIL~~
PRHDI Aleodoin1-o	~~FTARSKPFAVLPVT			SVASWLALS	LVCCWIN	
Alcadeini-a	DESG-HILAN	CCUDNCMTDC		WCVGFLVE	MATCINE.	
Alcadein2-g	A A A A A A A A A A A A A A A A A A A	STONSMIPS		TTSVCMLVEY		VPTAHOUFT~~
API.P1	~~~~DELAP	AGTGVSRE~~	~~~AVSGLLT	MGAGGGSLT	T.SMT.T.T.R	RKKPYCATS~
ADOER2	~~~NHSOHYANEDSKM	GSTVT~~~~~	AVIGITY	PTVVTALLC	ASGYLTWR	WKRKNTKS~~
CD43	PTTSTNASTVPFRNPD	ENSR	~~~GMT.PVAVI.	VALLAVIVL	VALLEN	~RROKRRTGA~
CADH5	~~~EOGEFTFCED~~M	AAOVGVSTO~		LLCTLTTV	TTTTT	~RRI.RKOARA~
CDH1	~~~~VEAGL	OIP~~~~~~	~~~AILGILG	GILALLILI	LLLETR	~RRAVVKEPL~
CSF1R	FIPISAGAHTHPPDEF		~~~~ VVVAC	MSIMALLLL	LLLLYK	KOKPKYOV~~
DAG1	~~~EAPPTEVPDRDPE	KSSEDD~~~V	YLHTVIPAVV	VAAILLIAG	IIAMICYR	~KKRKGKLTL~
IL1R2	~~~FKCVVHNTLSFQT	LRTTVKE~~A	SSTFSWGIVL	APLSLAFLVI	LGGIWMHR	~RCKHRTGKA~
IL6RA	~~~~~ RDSANATSLP	VODSSSVPLP	······································	GGSLAFGTLI	LCIAIVIR	FKKTWKLRA~~
JAG1	~~~~LIAAVAEVRVQ	RRPLKNRTD~	FLVPLLSSVL	TVAWICCLV	TAFYWCIR	~KRRKPGSHT~
LRP2	~~~~GYTGKYCEMAFS	KGISPGTT~~	~~AVAVLLTIL	LIVVIGALA	LAGFFHYR	~RTGSLLPAL~
LRP6	~~~~CSDKSDELDCY	PTEEPAPQ~~	ATNTVGSVIG	VIVTIFVSG	IVYFICC R	MLCPRMKGD~~
SDC1	~~ VD QGATGASQG~~~	~LLDRKE~~~	~~VLGGVIAGG	LVGLIFAVC]	LVGFMLYR	MKKKDEGS~~~
SDC2	~~~ EEDTNVYTEKH SD	SLFKRTE~~~	~~VLAAVIA <mark>G</mark> G	VIGFLFAIF]	LILLVYR	MRKKDEGSY~~
SDC3	~~~GSSAAQLPQK~~~	SILERKE~~~	~~VLVAVIV <mark>G</mark> G	VVGALFAAF	LVTLLIYR	MKKKDEGSY~~
PAM	~~~~SS ELQKMQEKQK	LIKEPGS~~~	~GVPVVLITT	LTAI BAAAT	LAIAIFI <mark>R</mark>	WKKSRAFGD~~
PCDA4	ASSRALVGAVGPDAAL	VDVN ~~~~~~	~~~VYLIIAIC	AVSSLLVLT]	LLYTAIR	CSALPTEGA~~
PTPRF-LAR	~~ DEIVVQVTPAQQQ ~	~~~ EEPE ~~~	~~~ MLWVTGPV	LAVILIILIV	/IAILLE <mark>K</mark>	~RKRTHSPSS~
PTPRZ	~~~SNSSHESRIGLAE	GLESEKK~~~	AVIPLVIVSA	LTFICLVVLV	/GILIYWR	~KCFQTAHFY~
ROBO1	~~~~VSPEDQVS	LAQQISDVVK	OP~~AFIAGI	GAACWIILM	/FSIWLY <mark>R</mark>	HRKKRNGLT~~
SCN1B	~~~~~ KIHIEVVDK	ANRDMASIVS	E~~~IMMYVL	IVVLTIWLVA	YEWI ACAK	~KIAAATETA~
SORL	~~~~~ <mark>ELGSGADASA</mark>	TQAARSTD~~	~VAAVVVPIL	FLILLSLGV	FAILYIK	HRRLQSSFT~~
TYR	~~~~PDSFQDYIK	SYLEQASRIW	S~WLLGAAMV	GAVLTALLA	JLVSLLCR	HKRKQLPEE~~
TYRP1	~~~~ DNLGYTYEIQW	PSREFSVPE~	~~~IIAIAVVG	ALLLVALIF	FASYLIR	ARRSMDEAN~~
TYRP2	~~~~YSYAIDLPV	SVEETPGWPT	T~~LLVVMGT	LVALVGLFV	LAFLQYR	~RLRKGYTPL~
VASN	~~~~PPAVHSNHAPVT	QAREGNLP~~	~~~LLIAPALA	AVLLAALAA	/GAAYCVR	~ <mark>RGR</mark> AMAAAA~
VGFR1	~~~~SVESSAYLTVQ	GTSDKSNLE~	~~LIT~~LTC	TCVAATLFW	LLTLFIR	~KMKRSSSEI~
VLDLR	~~GGINVTTAVSEVSV	PPKGTS~~~~	~~~~ AAWAIL	PLLLLVMAA	/GGYLMWR	NWQHKNMKS~~

Transmembrane region

Figure S8: Sequences and properties of γ **-secretase substrates.** Alignment of the sequences of the 69 γ -secretase substrates with known cleavage sites. Only the last 20 residues of the extracellular domain (ECD; unless cutting at the known site) and the 10 N-terminal residues of the intracellular domain (ICD) are presented. The hydrophobic TM domain is indicated by the green bracket. Boxed arginine and lysine residues are the conserved positively charged residues at the TM junction site. The color code is the same as in Figure 7.



Figure S9: Normalized epsilon-cleavage assay activities (a) and amounts of Aβ40 (b) and Aβ42 (c). Data were normalized to relative protein expression in Figure 2b and Figure 3e, respectively.

Supplementary information, Table S1

 Table S1: Oligonucleotide primers used in this study.
 All primers were

 synthesized by Integrated DNA Technologies.

Primers used in this study					
name	sequences				
PS1-Guide-CDS1-F	CACCGTGGAAGTAGGACAACGGTGC				
PS1-Guide-CDS1-R	AAACGCACCGTTGTCCTACTTCCAC				
PS1-Guide-CDS2-F	CACCGATTATCTAATGGACGACCCC				
PS1-Guide-CDS2-R	AAACGGGGTCGTCCATTAGATAATC				
PS2-Guide-CDS1-F	CACCGTGAGCGGACGTCCCTAATGT				
PS2-Guide-CDS1-R	AAACACATTAGGGACGTCCGCTCAC				
PS2-Guide-CDS2-F	CACCGCGTGCTTCGCTCCGTATTTG				
PS2-Guide-CDS2-R	AAACCAAATACGGAGCGAAGCACGC				
PS1-P1	TTGCGGTCCTTAGACAGCTT				
PS1-Pindel	AATAGAGAACGGCAGGAGCAC				
PS1-P2	ACGACCACCATGCAGAG				
PS2-P1	CTGCCCAGGAGAGAAATGAG				
PS2-Pindel	TGACCGCTATGTCTGTAGTGG				
PS2-P2	TGTAGAAGCGCACAGACTTG				
hGAPDH-614F	TGGAAGGACTCATGACCACA				
hGAPDH-776R	TTCAGCTCAGGGATGACCTT				

Supplementary information, Table S2

Table S2: FAD-linked C99 mutations. List of FAD-linked APP mutations within C99 as listed in the Alzforum database (http://www.alzforum.org/mutations).

FAD-linked C99 mutations					
APP Mutations	corresponding				
	C99 position				
A673V	2				
H677R (English)	6				
D678H (Taiwanese)	7				
D678N (Tottori)	7				
E682K (Leuven)	11				
K687N	16				
A692G (Flemish)	21				
E693del (Osaka, E693∆, E693delta)	22				
E693G (Arctic)	22				
E693K (Italian)	22				
E693Q (Dutch)	22				
D694N (Iowa)	23				
L705V	34				
A713T	42				
T714A (Iranian)	43				
T714I (Austrian)	43				
V715A (German)	44				
V715M (French)	44				
I716F	45				
I716V (Florida)	45				
I716T	45				
V717F (Indiana)	46				
V717G	46				
V717I (London)	46				
V717L	46				
T719P	48				
L723P (Australian)	52				
K724N (Belgian)	53				