Supporting Information

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SI Methods

Cell Lines, cDNA Constructs, and Transfection. cDNAs encoding full-length human nicastrin (NCT) and NCT mutants were C-terminally tagged with CT11 tag (1). cDNAs encoding the entire ectodomain segment (ECD) or a region corresponding to exons 7–16 ("716"), exons 7–15 ("715"), residues 1–585 (NCT1-585), and residues 1–630 (NCT 1–630) were C-terminally tagged with 6xHis tag. HEK293 cells and HEK293S GnTI$^-$ cells (a kind gift from Kelly Moremen, University of Georgia, Athens, GA) (2) were stably transfected with NCT constructs using Lipofectamine and Plus reagent (Invitrogen). HEK293 cells, HEK293S GnTI$^-$ cells, and nicastrin knockout (NCT$^{-/-}$) fibroblasts (kindly provided by Philip Wong, Johns Hopkins University School of Medicine, Baltimore, MD) (3) were maintained in DMEM containing 10% (vol/vol) FBS, 1% (wt/vol) penicillin-streptomycin (PS) (Invitrogen). To examine γ-secretase activity, a mouse Notch 1 lacking the ECD segment (NΔE)-6xmyc construct (4) and either WT or mutant NCT constructs were used to transiently cotransfect NCT$^{-/-}$ fibroblasts for 48 h before detergent-solubilized cell lysates were prepared for analysis. To assess conversion of full-length PS1 into PS1 fragments, either WT or mutant NCT constructs were used, along with cDNAs encoding human presenilin enhancer (PEN)-2, anterior pharynx defective (Aph)-1aL, and PS1, to cotransfect NCT$^{-/-}$ fibroblasts for 48 h before analysis of detergent-solubilized extracts.

Western Blotting and Antibodies. Cells were lysed in buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA, and protease inhibitor mixture (Sigma). Protein concentrations were determined by bicinchoninic acid kit (Thermo Scientific). Equal amounts of protein lysates were resolve on SDS/PAGE and transferred to nitrocellulose membrane. After blocking, the membrane was sequentially incubated with primary and secondary antibodies, and the secondary antibodies were detected with electrochemiluminescence (ECL; PerkinElmer). $PS1_{NT}$ antibody (1) was used to detect full-length and N-terminal fragment of PS1. PNT-2 (1) antibody (a kind gift of Gopal Thinakaran, University of Chicago, Chicago, IL) was used for the detection of PEN-2 protein. CT11 antibody (1) was used to detect CT11-tagged NCT and PEN-2. Nicastrin (N-19) antibody (Santa Cruz) was used to detect endogenous NCT. 9E10 (Santa Cruz) was used to detect myc-tagged Aph-1aL and 6-myc–tagged mNΔE fragments. Anti-6xHis antibody (Rockland) was used to detect 6xHis-tagged truncated NCT. CTM1 polyclonal antibody (5) was used for the detection of full-length amyloid precursor proteins (APP) and APP-carboxyl-terminal fragments (CTFs). 26D6 monoclonal antibody (6) was used to detect APPs and Aβ.

Purification of Secreted NCT Fragments. HEK293S GnTI$^-$ cells stably expressing NCT ECD or 716 were cultured in DMEM containing 5% FBS and 1% PS. Conditioned medium was collected and filtered through a 0.45-μm filter. Secreted ECD and 716 were precipitated in 80% ammonium sulfate, and this fraction was dissolved and dialyzed overnight in 1× PBS (pH 7.5). Dialyzed samples were incubated with Ni-NTA beads (Invitrogen) and after extensive washing, bound ECD or 716 was eluted with buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 300 mM imidazole (pH 7.4), dialyzed against 50 mM phosphate buffer (pH 7.4), concentrated, and further purified using a Superdex 200 column (GE Healthcare) in phosphate buffer containing 20 mM phosphate (pH 7.4) and 150 mM NaCl. The purity of purified samples was assessed using Coomassie staining and Western blotting.

Generation of Anti-nicastrin Synthetic Antibodies. The synthetic antibody library built using the 4D5 Fab scaffold, selection methods, and hit characterization have been described previously (7). The ECD was purified from the conditioned medium of HEK293 cells, treated with Peptide: *N*-Glycosidase F (PNGaseF), and was biotinylated with sulfo-NHS-SS-biotin (Thermo Scientific). The target concentrations were 100 nM in the first round and 50 nM in the subsequent rounds.

Fab Expression and Purification. Fab2 and Fab12 expression was performed essentially as previously described (8). However, the transformants were first grown ~24 h at 30 °C in 1 L 2× yeast tryptone broth supplemented with 100 μg mL^{-1} ampicillin. The cells were harvested and resuspended in 1 L of CRAP Media (8) that was initially depleted of phosphate by addition of 100 mM $MgCl_2$ and 100 mM NH_4OH, sterile filtered, and pH-adjusted to 7.3. Fab2 and -12 were purified on a HiTrap Protein G HP column (GE Healthcare) using 20 mM sodium phosphate buffer and eluted with 0.1 M glycine·HCl (pH 2.7). The eluted Fabs were then loaded onto a Resource S column (GE Healthcare), washed with 50 mM sodium acetate buffer (pH 5.0), and eluted with a linear gradient of 50 mM sodium acetate buffer (pH 5.0) with 1 M NaCl. Fractions were verified by SDS/PAGE, pooled, and dialyzed to 20 mM sodium phosphate buffer (pH 7.4) with 150 mM NaCl.

Immunoprecipitation of Secreted NCT Fragments with Anti-NCT Fabs. Conditioned medium containing secreted NCT fragments were adjusted with 5× PBS to a final concentration of 1× PBS, 4 μg of purified Fab was added, and the mixture was incubated overnight. The Fab–antigen complexes were captured with protein L beads (Thermo Scientific) and detected using Western blotting with the anti-6xHis antibody. For immunoprecipitation of NCT from solubilized HEK293 cell membranes, the reaction mixture contained 0.25% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO).

In Vitro γ-Secretase Activity Assay. γ-Secretase complexes were purified from HEK293S cells that coexpress human PEN-2-CT11, Aph-1as, NCT-CT11, and TAP-tagged PS1 as described previously (9). Purified active γ-secretase was preincubated with either negative control Fab, a positive control antibody Mab5226A (10), Fab2, or Fab12 on ice for 1 h, and the γ-secretase activity was assayed using an APP (sb4, 1 μM final concentration) or Notch1 substrate (11, 12). Reactions were performed at 37 °C for 150 min, and the production of Notch intracellular domain (NICD) or Aβ peptides was determined using ECL or AlphaLISA signals (11, 12).

Immunostaining of HEK293 ANPP.8 Cells. HEK293 cells stably expressing Aph-1as, nicastrin-CT11, PS1, and PEN-2-CT11 (6) were seeded on chamber slides (Thermo Scientific/Nunc) and grown in DMEM with 10% FBS, 1% PS, and 200 μg of G418. After being washed three times with dialyzed phosphate buffered saline (DPBS) (Invitrogen), cells were fixed with prechilled methanol for 10 min on ice and rehydrated with DPBS. All subsequent incubations were carried out at room temperature in a humid covered dish to prevent drying. Cells were then blocked with 5% goat serum for 30 min. Fab stocks (0.02 mg/mL) were prepared in DPBS and incubated with cells for 1 h, followed by

three washes with DPBS. FITC-conjugated goat anti-human antibody was incubated with cells for 1 h in the dark. After three washes with DPBS, slides were mounted and bound antibodies visualized by under an inverted microscopy, and confocal images were acquired on a confocal laser-scanning microscope using FluoView software (Olympus). For NCT staining, fixed ANPP.8 cells were incubated with affinity-purified NCT54 antibody (6) at 10 °C for 45 min without prior fixation, as previously described (13). For analysis of PS1 localization, cells were fixed, permeabilized in 0.2% Triton X-100 (Tx-100), and incubated with affinity-purified $PS1_{NT}$ antibody in PBS/0.2% Tx-100/1% BSA overnight at 4 °C. After incubation with primary antibodies, double staining was carried out with Cy5-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. After washing with PBS, cells were mounted onto glass slides with Aqua Poly/Mount (Polyscience). Immunofluorescence staining was examined under an inverted microscopy using a 60× oil immersion objective. For lectin staining of cell-surface glycoproteins, FITC-conjugated *Vicia Villosa* agglutinin (VVA; Vector Laboratories; 10 μg/mL) were directly added during the secondary antibody incubation. Identical photomultiplicator values and parameters were used to acquire images to compare signals between cells.

PNGaseF and Endo H Treatment. For PNGaseF treatment, whole-cell detergent lysates or purified NCT fragments were first supplemented with glycoprotein denaturation buffer (0.5% SDS, 40 mM DTT) and incubated at room temperature for 10 min. Nonidet P-40 was then added to the mixture to a final concentration of 1%. G7 buffer [50 mM sodium phosphate (pH 7.5)] and PNGaseF (100 U) (New England Biolabs) were then added to the mixture and incubated for 1 h at 37 °C. For Endo H treatment, whole-cell detergent lysates or purified NCT fragments were supplemented with glycoprotein denaturation buffer and incubated at room temperature for 10 min, G5 buffer [50 mM sodium citrate (pH 5.5)] and Endo H (200 U) (New England Biolabs) were then added to the mixture and incubated for 1 h at 37 °C. At the end of the incubation, 4× Laemmli sample buffer was added to the mixture to quench the reaction. To generate ECD for Fab selection, ECD was treated with PNGaseF in G7 buffer without denaturation.

Circular Dichroism Spectroscopy (CD). Purified proteins (~300–400 μg/mL) were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. CD spectra for 200–250 nm in 0.5-nm steps with a 1-nm bandwidth and a 2-s averaging time were recorded at 25 °C on an AVIV 202 Spectrometer (AVIV Biomedical) using a 1-mm path-length quartz cuvettete. Triplicate wavelength scans were averaged, buffer subtracted, and converted to mean residual ellipticity as previously described (14).

Surface Plasmon Resonance (SPR). All measurements were carried out on either a BIAcore 2000 or 3000 instrument at 20 °C. His-tagged ECD from $GnTI^-$ cell medium was immobilized on a Ni-NTA chip (GE Healthcare), and Fabs were flowed in NTA running buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 50 μM EDTA, 0.005% Tween 20] following methods provided by the manufacturer. For epitope mapping experiments, His-tagged ECD was immobilized on the NTA surface, and pairwise injections of 2 μM Fabs were flowed at 10 μL/min. Data were double referenced by subtracting both a blank flow channel and buffer injections and processed using Scrubber2 (BioLogic Software). For kinetic measurements, either His-tagged ECD or 716 was immobilized on the NTA surface, and duplicate injections of threefold serial dilutions (2.5–200 nM) of each Fab were injected over three flow channels (each with varying 6His-NCT immobilization levels) at a flow rate of 30 μL/min. Each data set was double referenced, processed, and globally fit to a 1:1 bimolecular Langmuir model using Scrubber2. The reported K_d values represent an average of the global fits to each of the triplicate data sets.

Differential Scanning Fluorimetry (DSF). DSF measurements were performed on a BioRad-CFX384 real-time PCR instrument (Bio-Rad Laboratories). Fab and ECD samples, 1 μM each, were prepared in 50 mM sodium phosphate buffer (pH 7.4) with 150 mM sodium chloride and 4× (1/1,250 dilution of stock) Sypro Orange dye (Life Technologies) to a total sample volume of 25 μL. Thermal melts were performed by heating the samples 25 °C to 95 °C, increasing the temperature in steps of 0.5 °C/30 s. Wavelengths of 490 and 575 nm were used for excitation and emission, respectively. Samples were processed with the CFX software provided by the manufacturer.

Coimmunoprecipitation of γ-Secretase Complex Under Native Conditions. $NCT^{-/-}$ fibroblasts expressing human Aph-1aL-Myc-His, PS1, PEN-2-CT11, and either WT or L571 mutant NCT were lysed in a buffer containing 50 mM Tris·Cl (pH 8), 150 mM NaCl, 1% CHAPSO, 1 mM DTT, 5 mM EDTA, amd 1× protease mixture (Sigma). Cell lysates were precleared with protein A beads before 1.5 μL of anti-PS1 antibody was added to each reaction, and the mixture was incubated overnight in a cold room. The immunoprecipitated complexes were captured by protein A beads, which were washed three times with the lysis buffer. γ-Secretase complexes were released from the beads by incubation in Laemmli buffer at 37 °C for 15 min.

Coimmunoprecipitation of APP C-Terminal Fragments with γ-Secretase Complex. $NCT^{-/-}$ fibroblasts were transiently cotransfected with Aph-1aL-Myc-His, PS1, PEN-2-CT11, and one of the NCT variants, including full-length ECD, WT NCT, NCT L571P, and NCT ΔL571. The control cells were transfected with Aph-1aL-Myc-His, PS1, PEN2-CT11, and empty vector. Forty-four hours after transfection, cells were treated with 2 μM of L685,458 for 4 h. The cells were washed twice with DPBS (Invitrogen) at room temperature. Then the cells were incubated with 1 mM DSP [dithiobis(succinimidylpropionate); Pierce] in DPBS at room temperature for 20 min with gentle shaking. To terminate the reaction, cells were washed with DPBS containing 50 mM Tris (pH 7.4). Cells were then washed twice with cold DPBS and lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.25% SDS, and protease inhibitor mixture (Sigma). Protein G beads-cleared cell lysates were immunoprecipitated with the NCT-specific Mab5226A in a cold room overnight, and the immunoprecipitated protein complex was captured by protein G beads. After extensive washing with lysis buffer, captured protein complex was released from the beads with Laemmli buffer.

Reagents for Mass Spectrometry Analysis. Ammonium bicarbonate (NH_4HCO_3), iodoacetamide, and formic acid were purchased from Sigma-Aldrich. Trifluoroacetic acid and Tris(2-carboxyethyl)phosphine were obtained from Pierce (Thermo Fisher Scientific). Bovine trypsin and chymotrypsin were acquired from Roche Applied Science. HPLC-grade water and acetonitrile (ACN) were from Thermo Fisher Scientific. POROS 20 R2 beads were from Applied Biosystems, and C18 ZipTips were from Millipore (Thermo Fisher Scientific).

In-Gel Tryptic Digestion of Proteins. Coomassie-stained protein bands were excised from the gels and destained with 45% acetonitrile in 100 mM ammonium bicarbonate. The resulting gel slices were incubated with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride, alkylated by the addition of 50 mM iodoacetamide, and then, for the first gel preparation, digested in situ with trypsin (150 ng per band in 100 mM ammonium bicarbonate). For

the second gel, the bands were divided into two parts; one part was digested with trypsin and the second part with chymotrypsin (150 ng per band in 100 mM ammonium bicarbonate). The tryptic (or chymotryptic) peptides were extracted using POROS 20 R2 beads in 5% formic acid and 0.2% trifluoroacetic acid. The extracted peptides were concentrated and desalted using C_{18} ziptips and eluted with 0.1% trifluoroacetic acid in 40% acetonitrile, followed by 0.1% trifluoroacetic acid in 80% acetonitrile. The eluates were dried under vacuum using a Speed Vac concentrator. The dried-down eluate was reconstituted with 0.1% formic acid in 2:98 ACN:H_2O for liquid chromatography/tandem mass spectrometry (MS/MS) analysis.

Liquid Chromatography and Mass Spectrometry Methods. A Waters NanoAcquity UPLC system interfaced to a Thermo LTQ-Orbitrap mass spectrometer (Thermo Scientific) equipped with a nanospray ionization source was used for LC/MS/MS analyses. Reversed-phase LC was performed on a Waters BEH130 C_{18} column (100 μm × 100 mm, 1.7-μm particle size). Samples were trapped and washed in a Waters Symmetry C_{18} trap column (180 μm × 100 mm, 5-μm particle size) before separation in the capillary column. Gradient elution with 0.1% formic acid in water as solvent A and in ACN as solvent B, with solvent B raised from 1 to 50% in 30 min, then 50–85% in the next 10 min, was carried out. A flow rate of 0.5 μL/min was used.

The mass spectrometer was operated in positive mode with spray voltage at 2.1 kV, ion transfer tube voltage at 47 V, and ion transfer tube temperature at 135 °C. No sheath and auxiliary gas were used. Ion signal thresholds of 1,000 were used for MS/MS. A normalized collision energy of 35%, an activation of q = 0.25, and activation time of 30 ms were applied in MS/MS acquisitions. Data-dependent acquisition with automatic switching between MS and MS/MS modes was used. A full scan was acquired at a target value of 5×10^5 ions with resolution (R) of 60,000 at m/z 400. The lock mass option, using the polydimethylcyclosiloxane ion [PCM; protonated $(Si(CH3)_2O))_6$] at m/z 445.120025, was enabled for the MS scan for accurate mass measurement. The top eight most intense ions were selected for fragmentation in the LTQ. Collision-induced dissociation at a target value of 10,000 ions was used for fragmentation. The following dynamic exclusion settings were applied to precursor ions chosen for MS/MS analysis: repeat count 2, repeat duration 60 s, and exclusion duration 300 s.

Data Analysis. MS/MS spectra were searched against human IPI database (version 3.77) using Sequest (version 27, revision 11) Mascot (version 2.3.02; Matrix Science) and X! Tandem (2007.01.01.1, scaffold 3.1.2; Proteome Software) algorithms (15, 16). Searches were performed with full tryptic specificity (two missed cleavages); carbamidomethylated cysteine residues as static modification; deamidated asparagine and glutamine (+0.9840 Da), oxidized methionine, histidine and tryptophan (+15.9949 Da), and glycosylated asparagine (+203 Da) as differential modifications. A precursor mass error tolerance of 5 ppm and default product ion mass error tolerance of above searching algorithms were used (0.6 Da to 1.0 Da). Manual inspection of the tandem mass spectra and product ion lists was also conducted. Scaffold PTM (Proteome Software) was used to annotate posttranslational modification sites contained in MS/MS spectra. The program uses the ASCORE's probabilistic approach and scoring technique developed by Beausoleil et al. (17).

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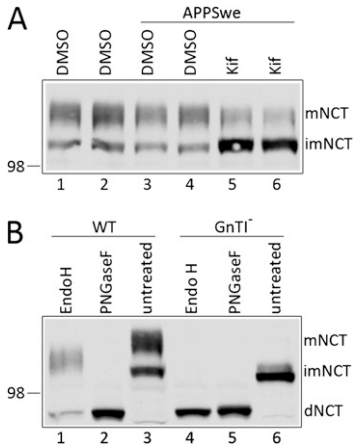

Fig. S1. Analysis of endogenous NCT glycoforms in HEK293, HEK293S, and HEK293S GnTI^{-} cells. (*A*) Analysis of endogenous NCT glycoforms in HEK293 cells (lanes 1–4) and cells treated with kifunensine (lanes 5 and 6). Duplicates are shown for each condition. (*B*) Analysis of endogenous NCT glycoforms in HEK293S cells (lanes 1–3) and GnTI^{-} cells (lanes 4–6). Whole-cell detergent lysates were left untreated (lanes 3 and 6) or treated with Endo H (lanes 1 and 4) or PNGaseF (lanes 2 and 5) and the resulting mixtures subject to Western blot analysis with NCT antibody N-19. dNCT, deglycosylated NCT; imNCT, immature NCT; mNCT, mature NCT.

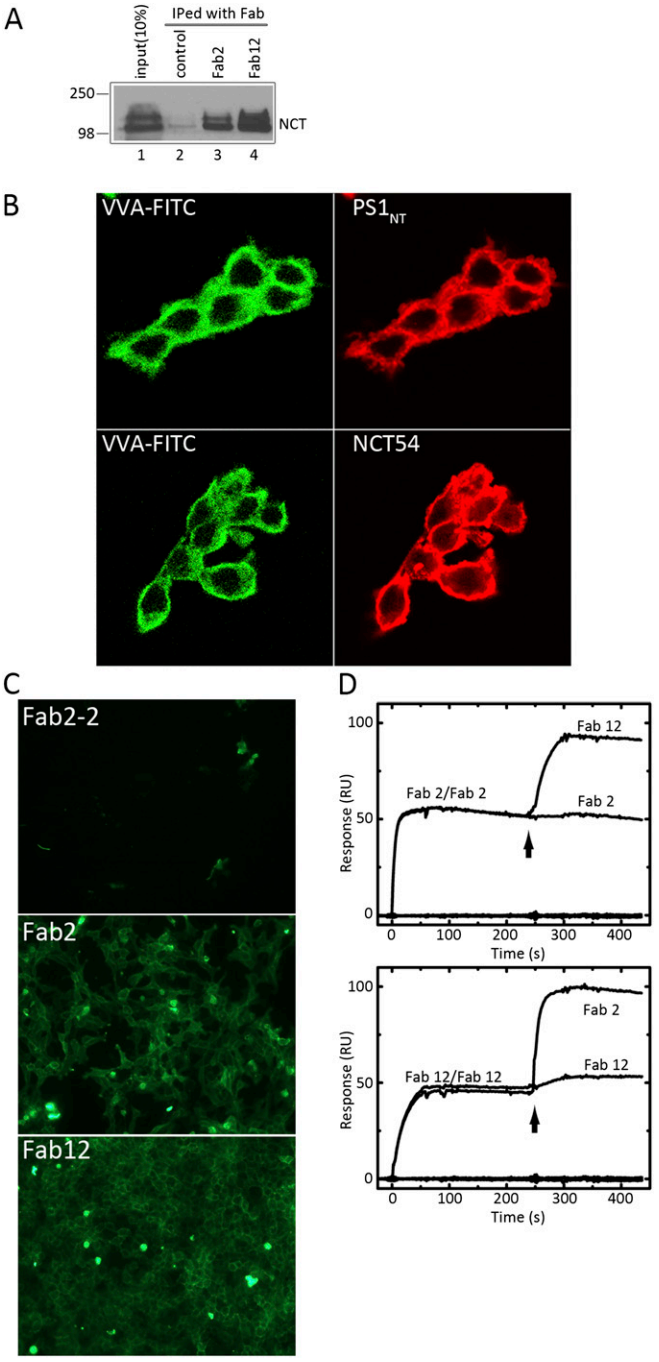

Fig. S2. Fab2 and Fab12 recognize full-length, CT11-tagged NCT. (*A*) Membrane fractions from HEK293S TAP-ANPP cells were immunoprecipitated with Fab 12, Fab 2, a NCT-specific Mab5226A, and a negative control Fab, and recovered NCT was detected by anti-CT11 antibody. (*B*) Accumulation of PS1 and NCT on the surface of ANPP.8 cells. VVA-FITC decorates plasma membrane glycoproteins (*Left*). Localization of PS1 (*Upper Right*) and NCT (*Lower Right*) is visualized by indirect immunofluorescence microscopy. (*C*) Immunostaining of HEK293 ANPP cells with Fab 2-2 (*Top*), Fab2 (*Middle*), and Fab12 (*Bottom*). (*D*) SPR result of sequential injection of Fab2 or Fab12 onto chips immobilized with ECD, demonstrating that Fab2 and Fab12 recognize different regions of ECD.

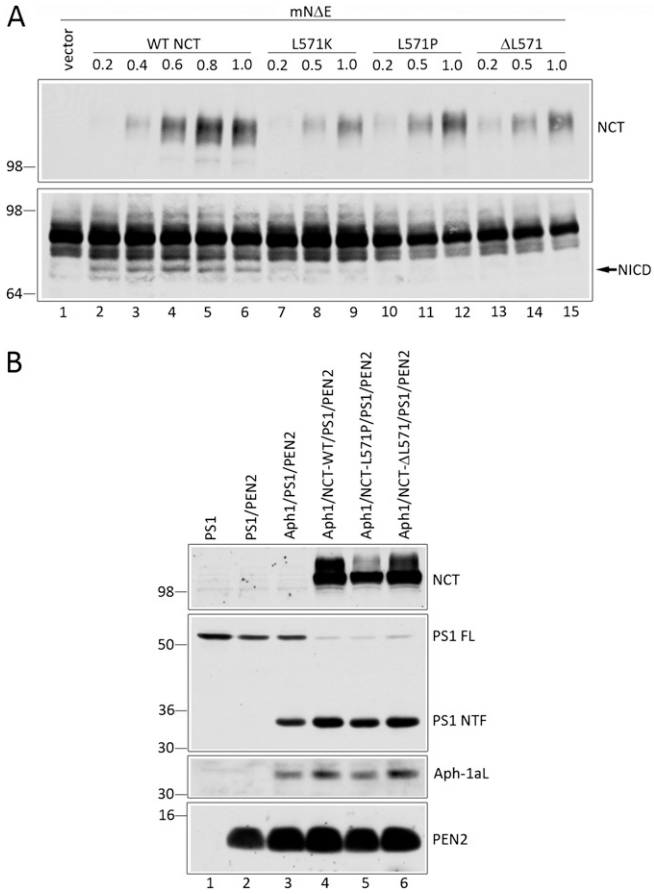

Fig. S3. L571 mutant NCT impairs γ-secretase activity, despite assembly into γ-secretase complexes. (*A*) NCT$^{-/-}$ cells transiently cotransfected with mNΔE and different amounts of WT or mutant NCT cDNAs. Expression of NCT (*Upper*) and mNΔE/NICD (*Lower*) are shown. (*B*) NCT$^{-/-}$ cells were transiently transfected with cDNAs encoding different γ-secretase components, singly or in combination, and lysates probed with antibodies specific for PS1, PEN-2, NCT, or Aph-1aL.

Table S1. Determination of *N*-linked glycosylation sites of purified ECD fragment by mass spectrometry analysis

		Identified in (Endo H-treated)		Identified in (PNGase F-treated)	
Amino acid residue	Reference	Trypsin digestion (gel 1)	Chymotrypsin digestion (gel 2)	Trypsin digestion (gel 1) deamidation	Chymotrypsin digestion (gel 2) deamidation
45	(1)	Yes	Yes	Yes	Yes
55	Potential	Yes		Yes	
187	(1)	Yes	Yes	Yes	Yes
200	Potential		Yes	Yes	
204	Potential	Yes	Yes	Yes	Yes
264	Potential	Yes	Yes	Yes	Yes
387	(1, 2)	Yes	Yes	Yes	
417	Potential	Yes	Yes	Yes	
435	Potential	Yes	Yes	Yes	Yes
464	Potential	Yes		Yes	
506	Potential	Yes	Yes	Yes	Yes
530	Potential	Yes	Yes	Yes	Yes
562	Potential	Yes	Yes	Yes	
573	Potential	Yes	Yes	Yes	
580	Potential	Yes	Yes	Yes	
612	(3)	Yes		Yes	

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