Experimental Procedures

Preparation of mouse tissue lysates A 24 week-old male mouse was sacrificed and the organs were removed (brain, liver, testes, spleen, stomach, kidney, bladder, heart, lung, small and large intestine), washed thoroughly in ice cold PBS. The tissues were cut into small pieces, homogenised in ice-cold lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween-20, 10% glycerol, 2 mM Na_3VO_4, 20 mM NaF, and protease cocktail inhibitor) with the potter homogenizer for 50 strokes at 4°C. The resulting homogenates were sonicated on ice and centrifuged 10,000 x *g* for 20 min to remove any insoluble material. Equal amounts of protein (25µg) from 10,000 x *g* supernatant fractions from different mouse tissue extracts were separated by SDS-PAGE and analyzed by immunoblotting as described above.

Fluorescent OST peptide assay The donor lipid-linked oligosaccharide (LLO) was prepared from yeast as previously described (1). The membranes from HeLa cells were prepared as described in the section of RNA interference and *in vitro* translation. The OST assay has been adapted from the previously published method by Kohda and colleagues (1). Briefly, 10µl of LLO in chloroform:methanol:H2O (10:10:3) was transferred to a 1.5ml eppendorf tube and dried in a SpeedVac for 15 min. A reaction mix was prepared consisting of 6µl LLO buffer (50mM Tris-HCl, pH 7.5, 10mM $MnCl_2$), 0.5µl Protease cocktail EDTA free mix, 2µl dH_20, 6µl membranes, and 0.5µl of 30µM N-terminal 5-FAM-peptide solution (5-FAM-Gly-Asn-Ser-Thr-Val-Thr) synthesised by Cambridge Bioscience (UK). The reaction was incubated for 3 hours at 30°C and stopped by adding 2.5µl of 5x SDS sample buffer and heated at 95°C for 5 min. The samples were separated by SDS-PAGE on a 15-25% gradient gel at not more than 23mA constant current. The gels were visualised using the LAS-3000 multicolour image analyzer (FujiFilm, UK), using the blue LED (460nm) illuminator and a Y515 filter.

RNA inference and in vitro translation 21 nucleotide duplexes corresponding to human DC2 (DC2#1, CTCTACTGTAAAGACTAAA; DC2#2, CGCATAACTTCGAACCTGA), KCP2 (KCP2#1, GACGCATGCGCATAGCTAA; KCP2#2, GACCCTGAATGTTCTATAA), STT3A, and STT3B (2) with symmetric 2 nucleotide 3' (2'deoxy) thymidine overhangs were synthesized and annealed (QIAGEN). A negative RNAi control, siCONTROL Risc-free siRNA from Dharmacon is a non-functional, non-targeting siRNA detecting for off-target effects resulting from transfection. HeLa cells (60% confluent) grown in $10cm^2$ dishes seeded 24 h prior to treatment were transfected with 130 µl of 20µM siRNA duplex using Oligofectamine (Invitrogen) as described (3). RNAi treated cells were incubated for 72 hrs, tunicamycin (20µg/ml) was added 12 hrs or overnight prior to preparation of semi-permeabilised cells as a source of ER membranes for translation or RT-PCR analysis of knockdown (4). A rabbit reticulocyte lysate system (Promega) was used for protein synthesis of a number of substrates for 60 min at 30°C in the presence of 0.75 µCi/µl [^{35}S] methionine and RNAi treated semi-permeabilised HeLa cells. Aurintricarboxylic acid (ATCA) was then added to a final concentration of 100µM to inhibit further initiation, and the samples were incubated at 30°C for 10 min. Membrane-associated products were isolated by centrifugation for 1 min at 16,000xg and washed by resuspension in KHM (20mM HEPES pH7.2, 110mM KOAc, 2mM MgOAc). All samples were incubated with 25 µl sample buffer for 10 min at 70°C and analyzed on SDS-polyacrylamide Tris-glycine gels. The resulting gels were dried and then visualised using autoradiography. Protein knockdown was analyzed by Western blotting either with rabbit polyclonal antisera specific for DC2, KCP2, or mouse monoclonal antisera for βactin as indicated.

RT-PCR Trizol reagent (Invitrogen) was used to isolate total RNA from treated HeLa cells. Using the first-strand cDNA synthesis, RNA was reverse transcribed into single-stranded cDNA with

avian myeloblastosis virus (AMV) reverse transcriptase and Oligo-p$(dT)_{15}$ according to manufacturers protocol (Roche Diagnostics). The reaction was stopped by incubation at 95°C for 5 min. PCR primers for DC2 (5'- agt gct cga atg tcc caa cc -3' and 5'- tca gat agc ccg gca gtt tc -3'), KCP2 (5'- acc gca ccc ggt tca gct cg -3' and 5'- gct ctt gcc tgt gac ctt gg -3') were designed and used for the PCR amplification with Taq DNA polymerase (Roche Diagnostics). The cycling conditions for DC2 and KCP2 started with a 3 min denaturation at 95°C, followed by 45 cycles of 1 min annealing at 58°C, 2 min extension at 72°C, and 45 sec denaturation at 95°C. To validate cDNA synthesis, a PCR for GAPDH (glyceraldehdye-3-phosphate dehydrogenase) was performed using sense (5'-gacccttcattgacctcaactaca-3') and antisense (5'-gtccaccaccctgttgctgtagcc-3') primers. The cycling conditions for GAPDH started with a 3 min denaturation at 95°C, followed by 45 cycles of 1 min annealing at 60°C, 2 min extension at 72°C, and 30 sec denaturation at 95°C. The PCR products were separated on 1% agarose gel and visualised by ethidium bromide staining.

NICD generation using EDTA treatment HeLa cells were treated with RNA duplexes specific for DC2, KCP2, a siRisc free control (siRF) or mock transfected (Mock). Two days after siRNA treatment, Notch1 was transfected and incubated for a further 18 h. On day 3, the cells were washed once with HBSS buffer (without Ca or Mg) and incubated in 5mM EDTA/HBSS buffer for 15 min at 37°C. For γ-secretase inhibition, cells were treated with 1 μM L-685,458 for 4 h prior to and during EDTA incubation.

Transient Expression and Co-immunoprecipitation SH-SY5Y cells were maintained in RPMI640 medium supplemented with 10% fetal bovine serum, 5 mM glutamine, and grown at 37 °C with 5% CO2. The cells were transfected with V5-tagged versions of DC2 and KCP2 cloned into the mammalian expression vector pDNA5/FRT/TO, denoted DC2V5 and KCP2V5, using JetPEI (Ozyme); harvested 18 h after transfection; rinsed twice with phosphate-buffered saline; and lysed in 1% CHAPSO immunoprecipitation buffer (1% (w/v) CHAPSO, 140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride for 1 h on ice. The sample was centrifuged at ~13,000 x g for 10 min to pellet nuclei and non-solubilized material, the supernatant was removed, and immunoprecipitated using rabbit polyclonal antibodies against PS1, PEN-2, Nicastrin, APP, SPC25, DC2 or KCP2. The immunoprecipitated material together with a sample of the input lysate (one-tenth) was resolved by SDS-PAGE, and the resulting gels were analyzed by Western blotting with a mouse monoclonal anti-V5 antibody.

Indirect Immunofluorescence microscopy Cells grown on glass coverslips were fixed using methanol or 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 and 0.05% SDS. For PS1 localisation studies, SH-SY5Y cell line was treated with siRNA duplexes for DC2, KCP2 or mock transfected (Mock) followed by transfection with full length APP-695 (APP) and/or incubated for 5 hours with 5μg/ml brefeldin A. Triple immunofluorescent staining was performed using a rabbit polyclonal antibody against PS1, a mouse monoclonal against p58, and a sheep polyclonal against Sec61β. Anti-mouse IgG-Alexa-350, anti-rabbit IgG-Alexa-488, and anti-sheep IgG-Alexa-594 conjugated antibodies (Invitrogen). For DC2, KCP2 and Ribophorin I localization studies, HeLa cells were used and the secondary antibody was anti-rabbit IgG-Alexa Fluor 488. Fluorescent images were obtained using a charge-coupled device camera (Photonic science) driven by Visiolab 2000 software (Biocom).

Supplementary Figure legends

Figure S1 γ-secretase cleavage of Notch. A. HeLa cells treated with siRNA duplexes for DC2 (siDC2), KCP2 (siKCP2), a siRisc free control (siRF) or mock transfected (Mock) for 2 days followed by transfection of human Notch1 for 18 h. To analyse γ-secretase cleavage (S3) of Notch1. Notch cleavage was stimulated by the addition of 5mM EDTA treatment. HeLa cells were treated with 1μM L685,458 for 4 h prior to and during EDTA incubation. Western blots were performed after treatments with antibodies against DC2, KCP2, full length Notch1 (αNotch FL), Notch intracellular domain (αNICD) and β-actin. B. The amount of NICD present in the lysate samples was calculated. The data shown represents an average from three independent experiments with the standard error. Levels of NICD that differ from the control (Mock) with a significance of $P<0.001$ (***) are indicated by asterisks.

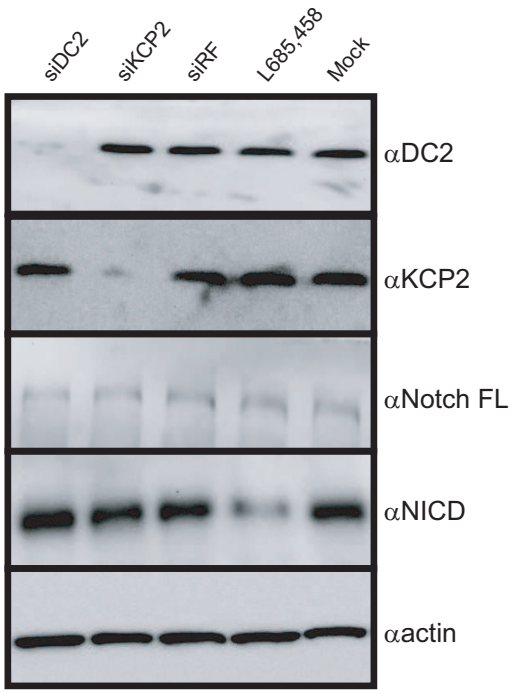
Figure S2 Cellular localization of the γ-secretase. HeLa cells grown on coverslips were treated with siRNA duplexes for DC2 (siDC2), KCP2 (siKCP2), or mock transfected (Mock) for 2 days followed by transfection of human PS1 for 18 h. On day 3, HeLa cells were also incubated for 5 hours with 5μg/ml brefeldin A (BFA). All samples were fixed and triple stained using antibodies against p58 (top row, blue), PS1 (second row, green) and Sec61β (third row, red). Merged images are shown in the bottom row. Bar, 20 μm.

References

1. Kohda, D., Yamada, M., Igura, M., Kamishikiryo, J., and Maenaka, K. (2007) *Glycobiology* **17**, 1175-1182
2. Wilson, C. M., and High, S. (2007) *J Cell Sci* **120**, 648-657
3. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001) *EMBO J* **20**, 6877-6888
4. Wilson, R., Allen, A. J., Oliver, J., Brookman, J. L., High, S., and Bulleid, N. J. (1995) *Biochem J* **307 (Pt 3)**, 679-687

Figure S1

A.


siDC2
siKCP2
siRF
L685,458
Mock
αDC2
αKCP2
αNotch FL
αNICD
αactin

B.

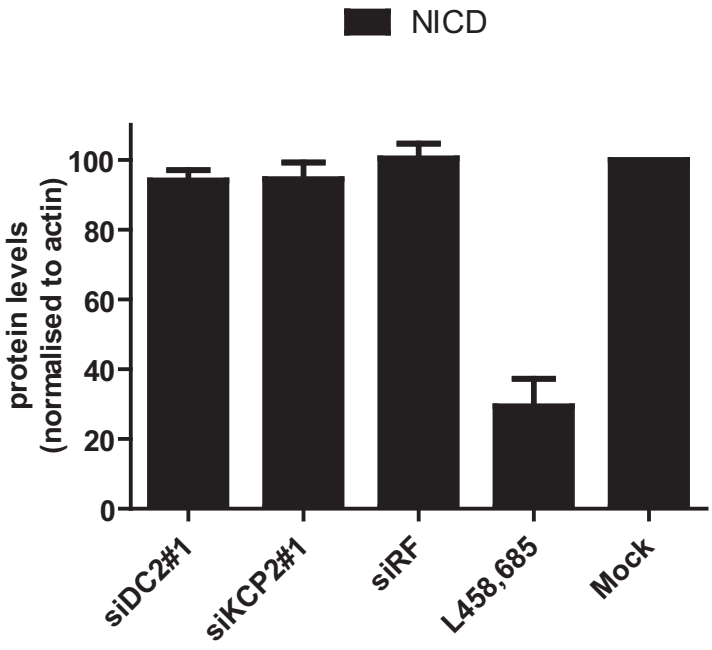

NICD
protein levels
(normalised to actin)
100
80
60
40
20
0
siDC2#1
siKCP2#1
siRF
L458,685
Mock

Figure S2