

Supplementary Data

Caveolin-1 Regulates γ -Secretase-Mediated A β PP Processing by Modulating Spatial Distribution of γ -Secretase in Membrane

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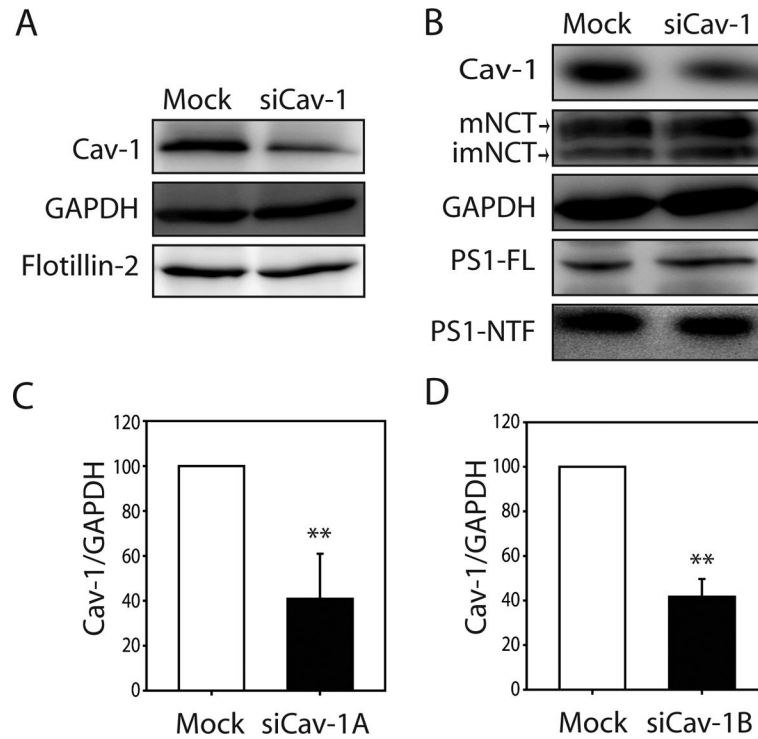
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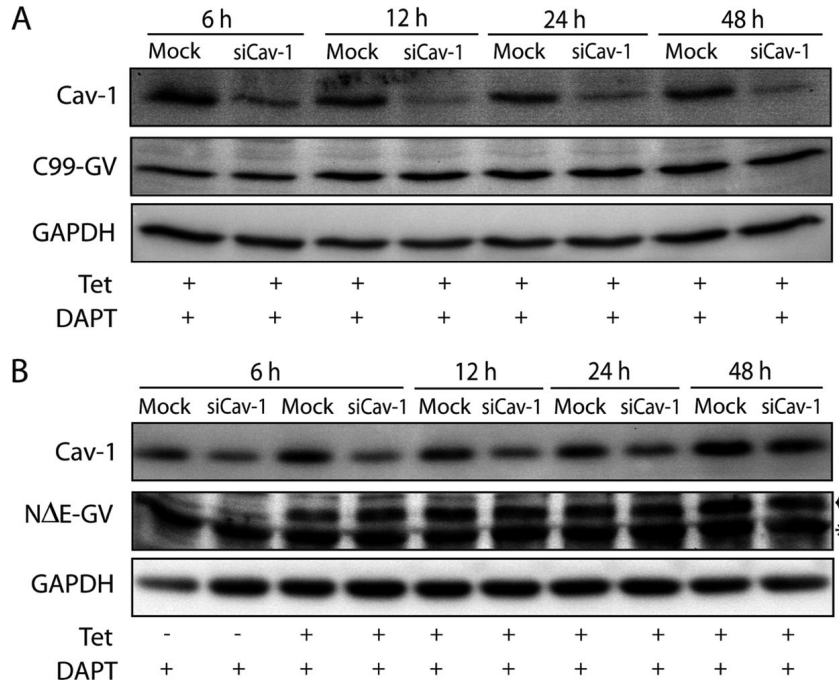
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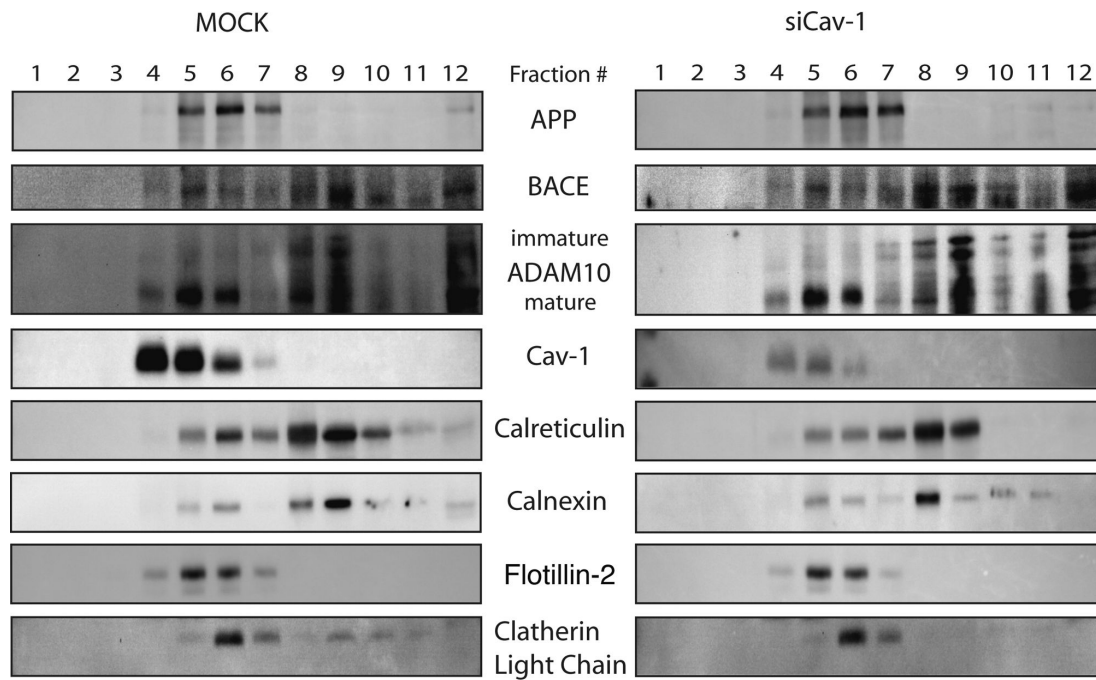
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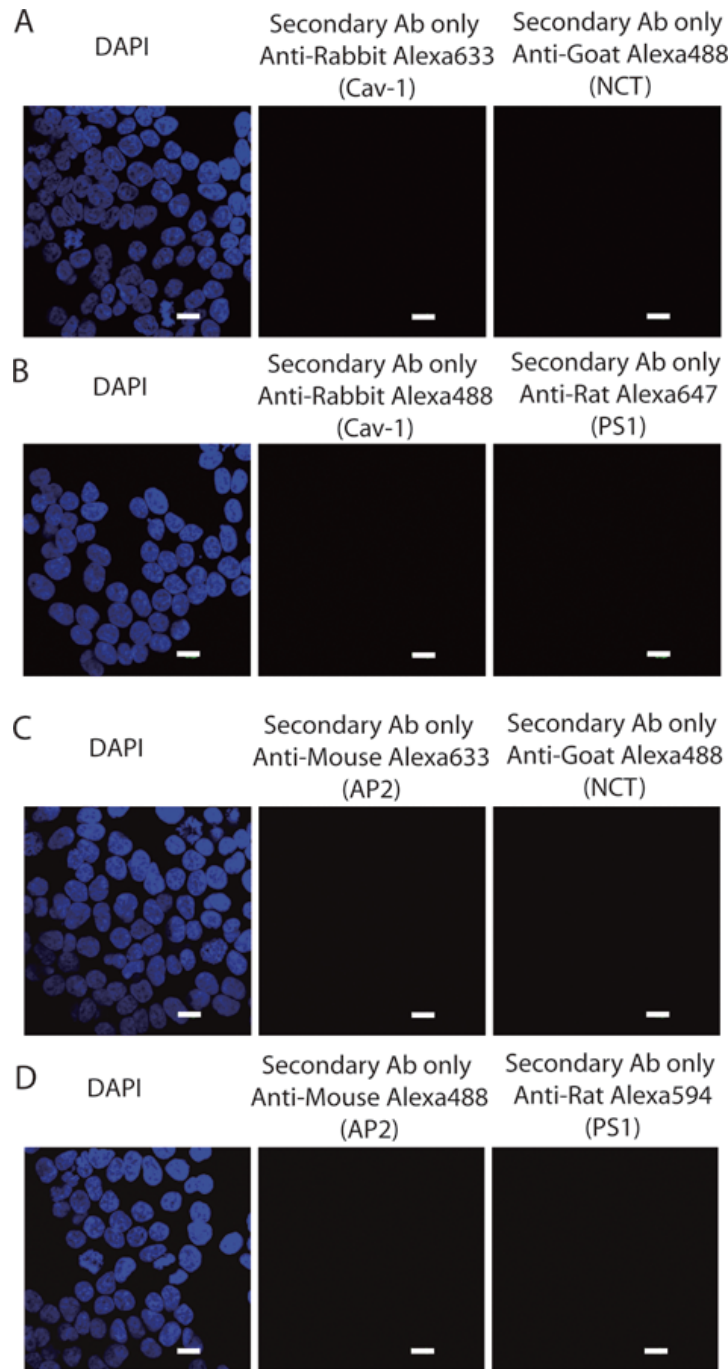
Supplemental Fig. 1. RNAi-mediated downregulation of caveolin-1 does not disrupt the integrity of lipid raft, nor the expression, maturation, and formation of functional γ -secretase complex. **A**) T20 cells were transfected with nonspecific siRNA (Mock) or siCav-1 for 48 h. The levels of flotillin-2 and caveolin-1 were determined using anti-flotillin-2 and anti-caveolin-1 antibodies (bottom and top panel). The levels of GAPDH were determined as loading control (middle panel). **B**) Lysates of mock- and siCav-1-transfected T20 cells were analyzed by SDS-PAGE and Western blotting with goat anti-nicastrin polyclonal antibody (for mature and immature NCT, mNCT and imNCT), anti-PS1 loop antibody (for full-length PS1, PS1-FL), anti-PS1 monoclonal antibody (PS1-NTF), anti-caveolin-1 antibody (Cav-1), or/and anti-GAPDH antibody (for loading control). **C, D**) The knockdown efficiency of two independent siRNAs targeting caveolin-1 (siCav-1A and siCav-1B) was determined in T16 cells. Quantitative results are expressed as the mean \pm S.D. from three independent experiments and analyzed by Student's t-test. * $p < 0.01$.



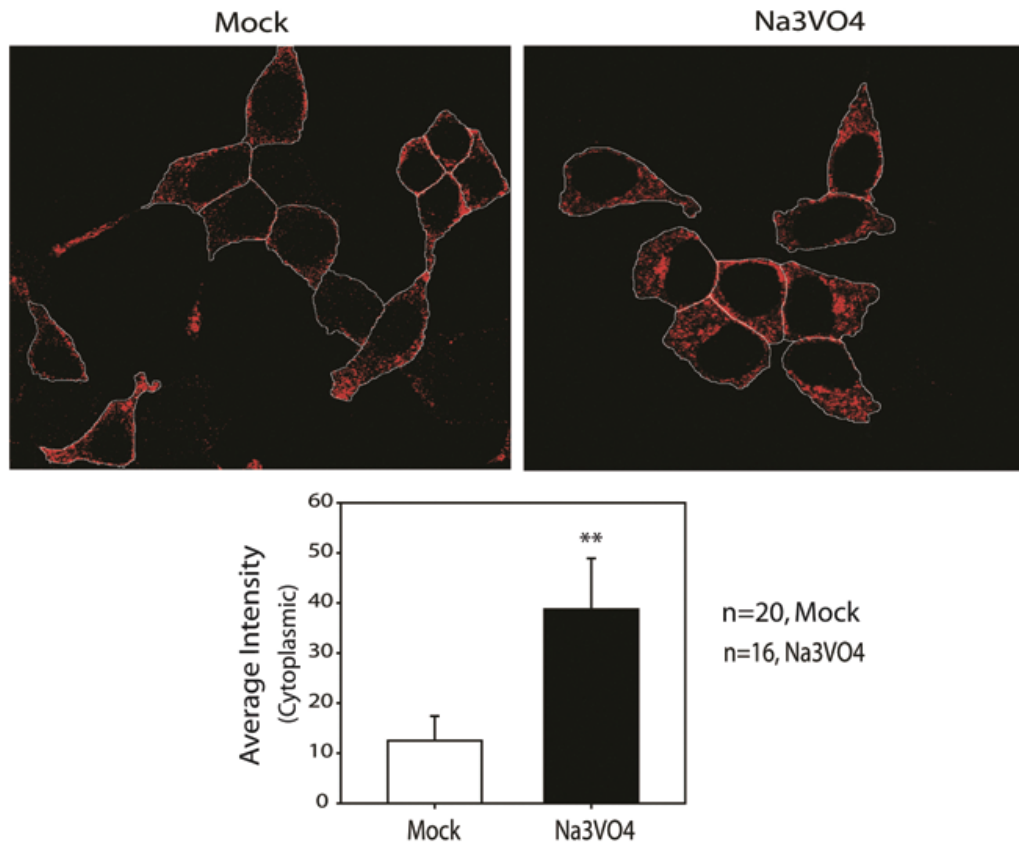
Supplemental Fig. 2. The down-regulation of caveolin-1 does not affect the stability of γ -secretase substrates A β PP-C99 and NΔE. A) T20 cells were transfected with a nonspecific scrambled siRNA (Mock) or siCav-1 for 24 h. Transfected cells were incubated with fresh medium containing 1 μ g/ml tetracycline and 10 μ M DAPT at 37°C for various intervals. Clarified lysates containing equivalent amounts of proteins were analyzed by SDS-PAGE and Western blotting with anti-caveolin-1 (Cav-1, top panel), anti-Gal4 (C99-GV, middle panel), and anti-GAPDH (loading control, bottom panel) antibodies. B) NGT cells were transfected with a nonspecific scrambled siRNA (Mock) or siCav-1 for 24 h. Transfected cells were incubated with fresh medium containing 1 μ g/ml tetracycline and 10 μ M DAPT at 37°C for various intervals. Clarified lysates containing equivalent amounts of proteins were analyzed by SDS-PAGE and Western blotting with anti-caveolin-1 (Cav-1, top panel), anti-Gal4 (NΔE-GV, middle panel), and anti-GAPDH (loading control, bottom panel) antibodies. Arrow to the right of middle panel denotes the tetracycline-inducible expression of NΔE-GV, while asterisk a non-specific protein band.



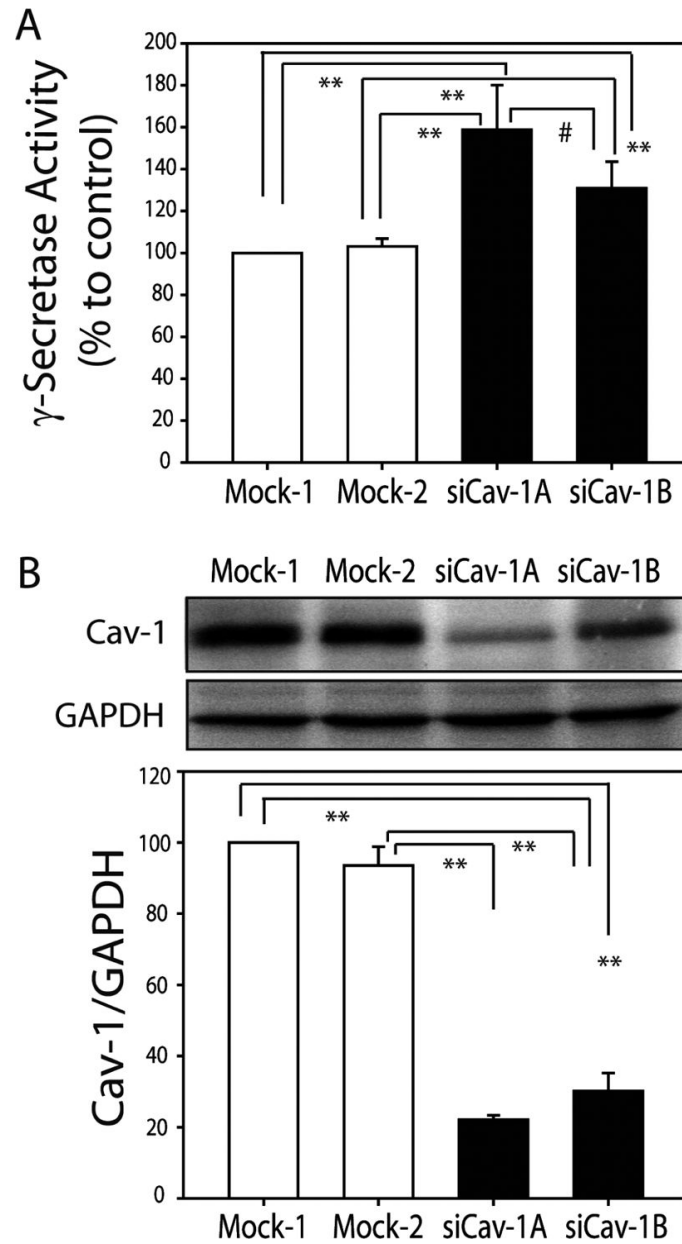
Supplemental Fig. 3. The distribution of A β PP-processing α -/ β -secretases, and compartment-specific marker proteins is not affected by caveolin-1 knockdown. Clarified lysates of siCav-1 and mock-transfected T16 cells were prepared using a lysis buffer containing 20 mM CHAPSO at 4°C. Postnuclear supernatants of transfected cells containing equivalent amounts of proteins were subject to discontinuous sucrose density gradient fractionation. Proteins collected in individual fractions were precipitated by TCA and analyzed by SDS-PAGE and immunoblotting. The distribution of A β PP, BACE (β -secretase), ADAM10 (α -secretase), calnexin (ER), flotillin-2 (lipid raft), and clathrin light chain (clathrin-coated vesicles), in different fractions were analyzed by specific antibodies. The majority of A β PP was localized to clathrin enriched fractions 6 and 7. The knockdown of caveolin-1 resulted in a slight increase in the level of CCV-resident A β PP.



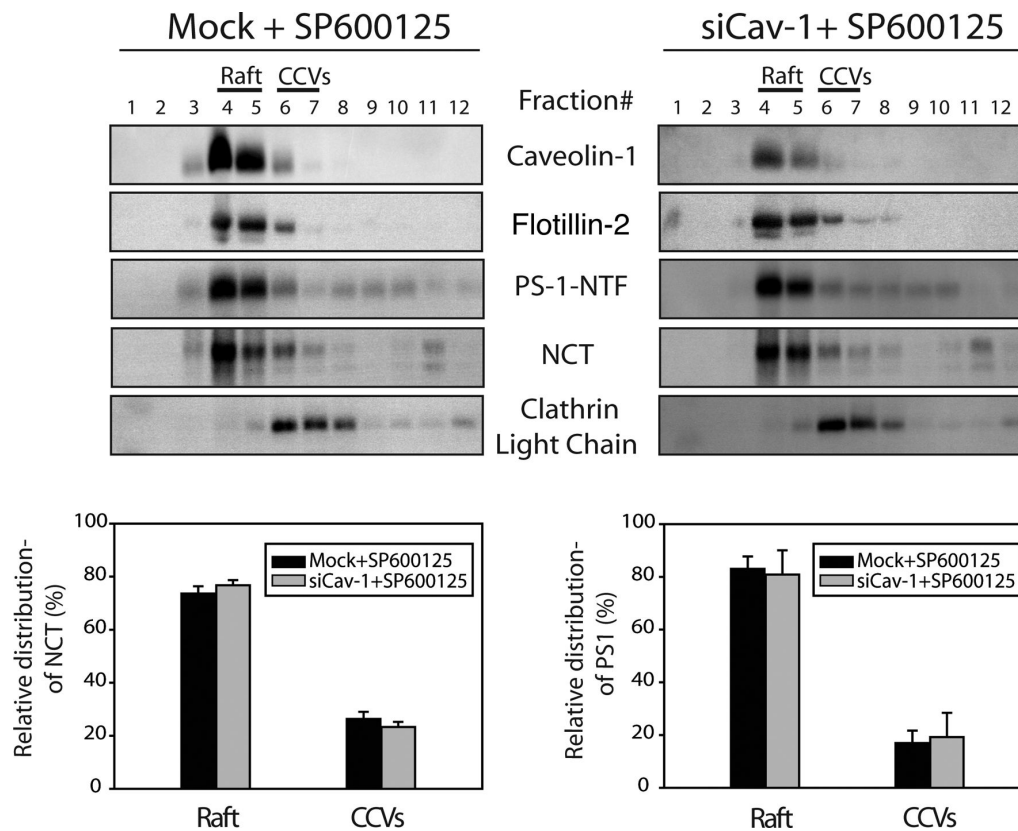
Supplemental Fig. 4. Fluorophore-conjugated secondary antibodies do not show nonspecific immunofluorescence signals in confocal microscopy. T20 cells were incubated with the combination of secondary antibodies in the absence of primary antibodies as specified in Figs 7 and 8 of the main text. DAPI staining was used for the visualization of cell nuclei of fixed cells. A) The secondary antibodies in combination were Alexa 633-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-goat IgG, the same combination of secondary antibodies as that in Fig. 7B for co-staining caveolin-1 and NCT. B) Alexa 488-labeled anti-rabbit IgG and Alexa 647-labeled anti-rat IgG were used as the same set of secondary antibodies in Fig. 7C for visualizing caveolin-1 and PS1. C) The combination of secondary antibodies were Alexa 633-conjugated anti-mouse IgG and Alexa 488-conjugated anti-goat IgG as used in Fig. 8A for co-staining AP2 and NCT. D) Alexa 488-labeled anti-mouse IgG and Alexa 594-labeled anti-rat IgG were used as shown in Fig. 8B for co-staining AP2 and PS1. Scale bar, 15 μ m.



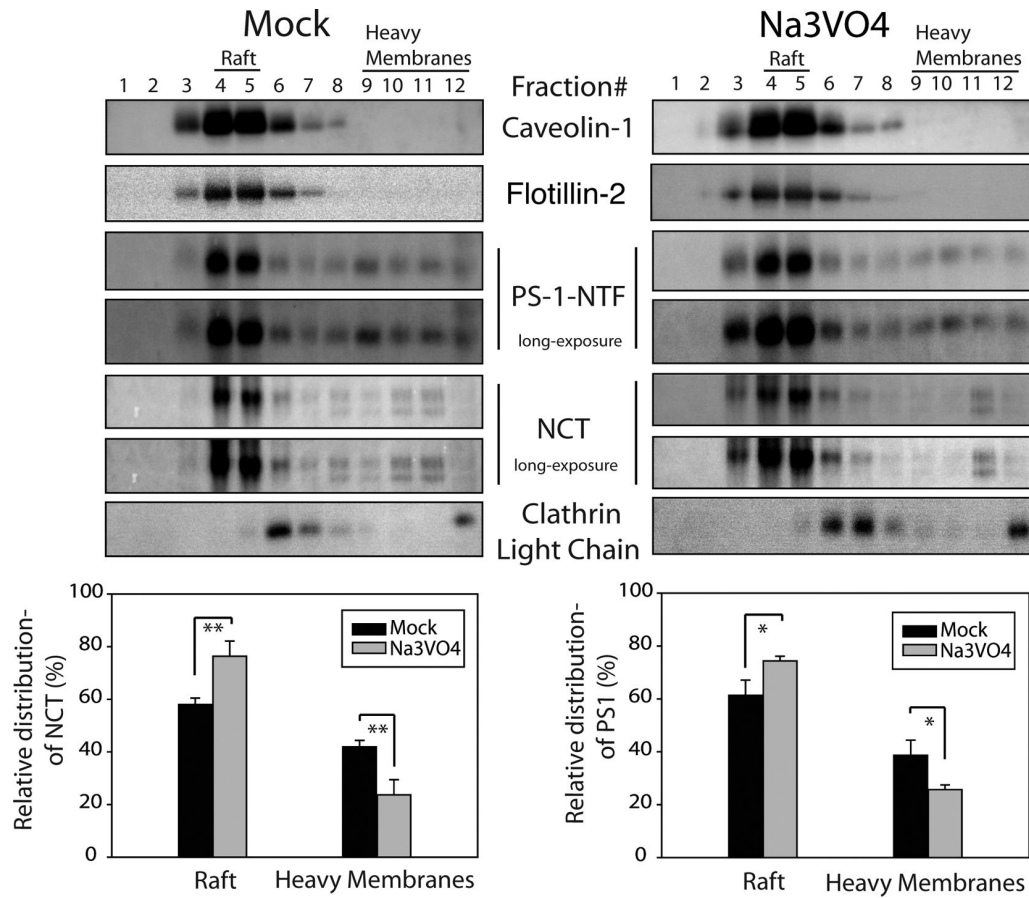
Supplemental Fig. 5. Treatment with sodium orthovanadate enhances overall caveolae-mediated endocytosis. T20 cells were treated with vehicle alone (0.1% DMSO, Mock) or 10 μ M sodium orthovanadate (Na_3VO_4) at 37°C for 1.5 h, and treated cells were fixed, permeabilized, and immunostained for caveolin-1 with the primary rabbit anti-Cav-1 and secondary Alexa 633-goat anti-rabbit IgG antibodies. Internalization of caveolin-1-labeled vesicles, indicative of caveolae-mediated endocytosis, was defined by drawing an area under the plasma membrane (white line) of each cell on the confocal image, and pixels of internalized vesicles within cytoplasmic area were quantified by using MetaMorph Offline 7.5.1.0 Image Analysis System (Molecular Devices). In this representative experiment, 20 DMSO-treated (Mock) and 16 orthovanadate-treated cells were analyzed. Data are shown as the average density (\pm S.D.) of cytoplasmic caveolin-1-specific pixels per cell and analyzed by Student's t-test. ** $p < 0.01$.



Supplemental Fig. 6. Two independent scrambled siRNAs do not affect the basal levels of γ -secretase activity. A) T20 cells were transfected with 50 pmol of either a nonspecific oligonucleotide (Silencer Negative Control 1, catalogue no. AM4611, Ambion) (Mock-1), a negative control siRNA (Bio Basic Inc, Markam, Canada) (Mock-2), siCav-1A, or siCav-1B. The sequences of nonspecific Mock-2 siRNA were as follows: 5'-UUC UCC GAA CGU GUC ACG UTT-3' (sense strand) and 5'-ACG UGA CAC GUU CGG AGA ATT-3' (antisense strand). The γ -secretase activity in the total cell lysates was determined after 48 h. B) Levels of caveolin-1 and GAPDH were resolved by using SDS-PAGE and Western blotting. Levels of caveolin-1 were similar in two mock-transfected samples (Mock-1 and Mock-2), while both siCav-1A and siCav-1B were effective on inducing significant down-regulation of caveolin-1. Quantitative results are expressed as the mean \pm S.D. from three independent experiments and analyzed by Student's t-test. * $p < 0.01$.



Supplemental Fig. 7. JNK activation is essential for the redistribution of γ -secretase into clathrin-enriched fractions in response to knockdown of caveolin-1. T20 cells were transfected with a scrambled siRNA (Mock) or siCav-1 for 24 h. Transfected cells were treated with 10 μ M of JNK inhibitor SP600125 for 24 h at 37°C, and clarified lysates were prepared using a lysis buffer containing 20 mM CHAPSO at 4°C. Postnuclear supernatants of transfected cells containing equivalent amounts of proteins were subject to discontinuous sucrose density gradient fractionation. Proteins collected in individual fractions were precipitated by TCA and analyzed by SDS-PAGE and immunoblotting for the distribution of Cav-1, Flotillin-2, PS1, NCT and clathrin light chain. Fractions 4 and 5 were enriched with caveolae/lipid raft markers (caveolin-1 and flotillin-2) and denoted as raft fractions, whereas fractions 6 and 7 were abundant with clathrin and denoted as CCVs. Bar graphs are the quantitative data for the average relative distribution of PS1 and NCT in caveolae/lipid rafts and CCVs from three independent experiments. The relative levels of γ -secretase constituents distributed in fractions 4 and 5 in proportion to their total levels in fractions 4–7 combined were denoted as raft-resident proteins, whereas those in fractions 6 and 7 as CCV-resident ones. Inhibition of JNK by SP600125 prevented the redistribution of PS1 and NCT into CCVs in siCav-1-transfected cells comparing to the results in Fig. 8. The distribution of PS1 and NCT in mock-transfected cells that are treated with either 0.1% DMSO alone (Fig. 8C, Mock) or SP600125 (Supplementary Fig. S7, Mock+SP600125) were consistent, suggesting that JNK modulates the distribution of γ -secretase in a caveolin-1-dependent pathway.



Supplemental Fig. 8. Treatment with sodium orthovanadate increases the co-localization of presenilin-1 and nicastrin with caveolin-1-enriched caveolae/raft domains. T20 cells were treated with or without Na₃VO₄ for 1.5 h at 37°C, and clarified lysates of cells were prepared using a lysis buffer containing 20 mM CHAPSO at 4°C. Postnuclear supernatants of transfected cells containing equivalent amounts of proteins were subject to discontinuous sucrose density gradient fractionation. Proteins collected in individual fractions were precipitated by TCA and analyzed by SDS-PAGE and immunoblotting for the distribution of Cav-1, flotillin-2, PS1, NCT and clathrin light chain. Fractions 4 and 5 were enriched with caveolae/lipid raft markers (caveolin-1 and flotillin-2) and denoted as raft fractions, whereas fractions 9 through 12 with heavier density were denoted as heavy membranes. Bar graphs are the quantitative data for the average relative distribution of PS1 and NCT in caveolae/lipid rafts and heavy membranes from three independent experiments, and analyzed by Student's t-test. **p* < 0.05; ***p* < 0.01. The relative levels of γ -secretase constituents distributed in fractions 4 and 5 in proportion to their total levels in fractions 4, 5, and 9–12 combined were denoted as raft-resident proteins, whereas those in fractions 9–12 as heavy membrane-resident ones. Vanadate-induced caveolae endocytosis led to significant increase in the redistribution of PS1 and NCT into caveolin-enriched raft domains from heavy membranes. Note that heavy membrane-localized PS1 and NCT are visible only after prolonged exposure.