Specific antibody binding to the APP<sub>672–699</sub> region shifts APP processing from α- to β-cleavage

S Li<sup>1,2,8</sup>, J Deng<sup>1,3,8</sup>, H Hou<sup>1,8</sup>, J Tian<sup>1</sup>, B Giunta<sup>4,5</sup>, Y Wang<sup>3</sup>, D Sawmiller<sup>1,4</sup>, A Smith<sup>6</sup>, PR Sanberg<sup>6</sup>, D Obregon<sup>1,4</sup>, T Mori<sup>7</sup> and J Tan<sup>1,4</sup>

Alzheimer’s disease (AD), a progressive neurodegenerative disorder that is the most common cause of dementia in the elderly, is characterized by the accumulation of amyloid-β (Aβ) plaques and neurofibrillary tangles, as well as a progressive loss of synapses and neurons in the brain. The major pertinacious component of amyloid plaques is Aβ, a variably sized peptide derived from the integral membrane protein amyloid precursor protein (APP). The Aβ region of APP locates partly within its ecto- and trans-membrane domains. APP is cleaved by three proteases, designated as α-, β-, and γ-secretases. Processing by β- and γ-secretase cleaves the N- and C-terminal ends of the Aβ region, respectively, releasing Aβ, whereas α-secretase cleaves within the Aβ sequence, releasing soluble APPα (sAPPα). The γ-secretase cleaves at several adjacent sites to yield Aβ species containing 39–43 amino acid residues. Both α- and β-cleavage sites of human wild-type APP are located in APP<sub>672–699</sub> region (ectodomain of β-C-terminal fragment, ED-β-CTF or ED-C99). Therefore, the amino acid residues within or near this region are definitely pivotal for human wild-type APP function and processing. Here, we report that one ED-C99-specific monoclonal antibody (mAbED-C99) blocks human wild-type APP endocytosis and shifts its processing from α- to β-cleavage, as evidenced by elevated accumulation of cell surface full-length APP and β-CTF together with reduced sAPPα and x-CTF levels. Moreover, mAbED-C99 enhances the interactions of APP with cholesterol. Consistently, intracerebroventricular injection of mAbED-C99 to human wild-type APP transgenic mice markedly increases membrane-associated β-CTF. All these findings suggest that APP<sub>672–699</sub> region is critical for human wild-type APP processing and may provide new clues for the pathogenesis of sporadic AD.

Cell Death and Disease (2014) 5, e1374; doi:10.1038/cddis.2014.336; published online 14 August 2014

Abnormal functioning and/or processing of amyloid precursor protein (APP), a type I membrane protein, has a pivotal role in the pathogenesis of Alzheimer’s disease (AD).<sup>1–3</sup> APP is cleaved by three proteases, designated as α-, β-, and γ-secretases (Supplementary Figure S1). The major fraction (>90%) of wild-type APP is proteolysed by α-secretase that cleaves wild-type APP between residues APP<sub>687</sub> and APP<sub>688</sub> within the amyloid-β (Aβ) sequence, releasing soluble APPα (sAPPα) and α-C-terminal fragment (α-CTF, C83). Only a minority (<10%) of all wild-type APP molecules undergo β-cleavage at the β-cleavage site (between residues APP<sub>671</sub> and APP<sub>672</sub>) generating sAPPβ and β-CTF (C99), the latter of which is subsequently processed by γ-secretase complex to generate a mixture of Aβ peptides primarily 40 or 42 residues in length (Aβ<sub>1-40</sub> or Aβ<sub>1-42</sub>).<sup>4,5</sup> The β-secretase cleaves APP in addition at a γ-site (between residues APP<sub>681</sub> and APP<sub>682</sub>) to generate C99 that is further processed by γ-secretase to produce truncated Aβ<sub>11-40</sub> species.<sup>6</sup>

Both α- and β-cleavage sites of wild-type APP are located in APP<sub>672–699</sub> region (the ectodomain of β-CTF, ED-β-CTF, or ED-C99; Supplementary Figure S1). Therefore, the amino acid residues within or near this region are definitely pivotal for wild-type APP function and processing. Previous studies have identified that mutation in ED-C99 region can affect the physiological processing of APP and contribute to pathologi­cal features of familial AD (fAD). For example, Swedish APP carrying APP<sub>670</sub> mutation (KM→NL) is cleaved by β-secretase over 50-fold more efficiently than wild-type APP.<sup>7</sup>

<sup>1</sup>Rashid Laboratory for Developmental Neurobiology, Silver Child Development Center, Department of Psychiatry and Behavioral Neurosciences, Morsani College of Medicine, University of South Florida, Tampa, FL, USA. 2Center for Translational Research of Neurology Diseases, First Affiliated Hospital, Dalian Medical University, Dalian, China. 3Department of Neurology, Daping Hospital, The Third Military Medical University, Chongqing, China. 4James A. Haley Veterans’ Hospital, Tampa, FL, USA. 5Neuromunology Laboratory, Department of Psychiatry and Behavioral Neurosciences, Morsani College of Medicine, University of South Florida, Tampa, FL, USA. 6Center of Excellence for Aging and Brain Repair, Department of Neurosurgery and Brain Repair, Morsani College of Medicine, University of South Florida, Tampa, FL, USA. 7Departments of Biomedical Sciences and Pathology, Saitama Medical Center and Saitama Medical University, Kawagoe, Japan.

*Corresponding author: J Tan, Rashid Laboratory for Developmental Neurobiology, Silver Child Development Center, Department of Psychiatry and Behavioral Neurosciences, Morsani College of Medicine, University of South Florida, 3515 E. Fletcher Avenue, Tampa, FL 33613, USA. Tel: +1 813 974 9326; Fax: +1 813 974 1130; E-mail: jtan@health.usf.edu

These authors contributed equally to this work.

**Abbreviations:** Aβ, amyloid-β; APP, amyloid precursor protein; AD, Alzheimer’s disease; ADAM10, a disintegrin and a metalloprotease 10; BACE, β-site amyloid cleaving enzyme; CHO/APP<sub>wt</sub>, Chinese hamster ovary cells stably transfected with human wild-type APP; CTF, C-terminal fragments; DMEM, Dulbecco’s modified Eagle’s medium; EC, entorhinal cortex; ED-β-CTF, ectodomain of β-C-terminal fragment; ELISA, enzyme-linked immunosorbent assay; fAD, familial AD; GSI, γ-secretase inhibitor; H, hippocampus; TgAPP<sub>wt</sub>, human wild-type APP transgenic; LRP1, low density lipoprotein receptor-related protein-1; LRP1-CT, cytoplasmic tail of LRP1; PBS, phosphate-buffered saline; RSC, retrosplenial cortex; sAD, sporadic AD; sAPPα, soluble APPα; TBS, Tris-buffered saline; WB, western blotting.

Received 19.5.14; revised 02.7.14; accepted 09.7.14; Edited by A Verkhratsky.
APP<sub>673</sub> mutation (A→V) and APP<sub>693</sub> mutation (E→G) can enhance Aβ production and accelerate formation of amyloid fibrils. APP<sub>688</sub> mutation (E→K) blocks APP β-site and shifts cleavage to α-site, thus increasing Aβ<sub>1–40</sub>/42 production. Although sporadic AD (sAD), the more common type of AD comprising 90 to 95% of all AD cases, lacks mutations in the APP gene, region-specific protein modifications within the ED-C99 region may affect wild-type APP processing similarly to APP gene mutations. For example, phosphorylation of ED-C99 at the threonine 687 (of APP<sub>770</sub> isoform, or corresponding threonine 668 of APP<sub>751</sub> isoform; Supplementary Figure S1) facilitates APP processing by γ-secretase. Therefore, the elucidation of potential influences of region-specific modifications, induced by either endogenous or exogenous molecules, on wild-type APP processing would be especially critical for clarifying the mechanisms underlying the pathogenesis of sAD.

To confirm this hypothesis, we used one mouse monoclonal antibody specifically recognizing ED-C99 (mAb<sub>ED-C99</sub>) with its epitope at APP<sub>674–679</sub> (Supplementary Figure S1). The influences of mAb<sub>ED-C99</sub> binding on human wild-type APP processing were evaluated in vitro using Chinese hamster ovary cells expressing human wild-type APP (CHO/APP<sub>wt</sub> cells) and cortical neurons derived from human wild-type APP transgenic (TgAPP<sub>wt</sub>) mice. The in vitro effects of ED-C99 binding with mAb<sub>ED-C99</sub> on wild-type APP processing were further evaluated and confirmed in vivo using TgAPP<sub>wt</sub> mice and 5xFAD transgenic mice (Tg6799 line).

**Results**

Specific binding of mAb<sub>ED-C99</sub> inhibits α- but promotes β-cleavage of human wild-type APP. Western blotting (WB) analysis demonstrated that, compared with IgG<sub>1</sub> isotype, 2 h treatment of CHO/APP<sub>wt</sub> cells with mAb<sub>ED-C99</sub> dose-dependently inhibits APP α-cleavage, as evidenced by the markedly decreased sAPP<sub>α</sub> and α-CTF levels (Figure 1a). In contrast, neither mAb22C11 (anti-APP 66–81 antibody) nor mAb2B3 (sAPP<sub>α</sub>-specific antibody) inhibited the α-cleavage of APP (Figure 1b). Most notably, mAb<sub>ED-C99</sub> shifted APP processing from α- to β-cleavage, as indicated by decreased sAPP<sub>α</sub> and α-CTF levels in combination with increased β-CTF production (Figure 1c). In addition, the cell culture media were collected from the separated primary cortical neuronal cells following 8 h treatment with mAb<sub>ED-C99</sub> or IgG<sub>1</sub> isotype control at 1.25 μg/ml for Aβ<sub>1-40</sub>/42 and sAPP<sub>α</sub>-ELISA. The results were presented as ng of Aβ<sub>40</sub>/42 or sAPP<sub>α</sub> per mg of total intracellular proteins (mean ± S.D.; ***P<0.001). These ELISA data are representative of three independent experiments with similar results.

**Figure 1** Treatment with mouse monoclonal-specific anti-ED-C99 antibody (mAb<sub>ED-C99</sub>) markedly inhibits α-cleavage but promotes β-cleavage of human wild-type APP. (a) Human wild-type APP stably transfected CHO (CHO/APP<sub>wt</sub>) cells were plated in 24-well plates at 5 x 10<sup>5</sup>/well and treated with mAb<sub>ED-C99</sub> at 0–1.25 μg/ml as indicated. (b) CHO/APP<sub>wt</sub> cells were treated with mAb<sub>ED-C99</sub>, mAb22C11 (m22C11, recognizes APP 66–81), or mAb2B3 (specifically and structurally recognizes sAPP<sub>α</sub>, but not full-length APP) antibodies, or isotype IgG<sub>1</sub> isotype control at 1.25 μg/ml. Immediately after 2 h treatment, cell supernatants were collected for western blotting (WB) analysis of sAPP<sub>α</sub> (using mAb2B3, upper panels) and Aβ secretion (using a monoclonal Aβ<sub>1–12</sub> antibody BAM10, mAbBAM10, middle panels); cell lysates were prepared for WB analysis of APP processing products (using a polyclonal anti-C-terminal APP 751/770 antibody, pAb751/770) and β-actin (internal control, lower panels). (c) Primary neuronal cells were cultured from cortical tissues of 1-day-old TgAPP<sub>wt</sub> mouse pups and seeded into 24-well-plates at 2 x 10<sup>5</sup>/well for 18 h. These primary neuronal cells were treated with mAb<sub>ED-C99</sub> or IgG<sub>1</sub> isotype control at 1.25 μg/ml for 2 h and then cell cultured media were collected for WB analysis of sAPP<sub>α</sub> (left upper panel) and secreted Aβ levels (left lower panel), and β-actin (right lower panel). These WB data are representative of four independent experiments with similar results. (d) In addition, the cell culture media were collected from the separated primary cortical neuronal cells following 8 h treatment with mAb<sub>ED-C99</sub> or IgG<sub>1</sub> isotype control at 1.25 μg/ml for Aβ<sub>1-40</sub>/42 and sAPP<sub>α</sub>-ELISA. The results were presented as ng of Aβ<sub>40</sub>/42 or sAPP<sub>α</sub> per mg of total intracellular proteins (mean ± S.D.; ***P<0.001). These ELISA data are representative of three independent experiments with similar results.
with clearly elevated β-CTF level (Figure 1b). However, mAbED-C99 did not increase Aβ production, as assessed using mAbBAM10 (recognizes Aβ1–12; Figures 1a and b, middle panels).

Consistent with these findings in CHO/APPwt cells, 2 h treatment of primary cultured cortical neurons derived from TgAPPwt mice with mAbED-C99 dramatically inhibited β-cleavage but enhanced β-cleavage of APP, as indicated by decreased levels of sAPPα and β-CTF as well as markedly increased β-CTF generation, compared with IgG1-treated control cells (Figure 1c). In addition, sAPPα levels were also significantly decreased with mAbED-C99 treatment (Figure 1d, right panel). However, as seen with CHO/APPwt cells, mAbED-C99 did not significantly alter Aβ1–40/42 productions (Figure 1c, lower panel) and their levels (Figure 1d, left panel). Altogether, these results indicate that specific binding of mAbED-C99 to the ectodomain of β-CTF (ED-C99) reduces β-secretase processing while increasing β-secretase processing of APP.

F(ab′)2 fragment of mAbED-C99 sufficiently inhibits β- but promotes β-cleavage of human wild-type APP. In living cells, the Fc fragment of antibody binds nonspecifically to cell surface Fc receptors. In order to determine whether nonspecific binding of mAbED-C99 to the cell surface Fc receptors is necessary to inhibit APP β-processing, we generated the F(ab′)2 fragment of mAbED-C99 that lacks the Fc fragment. Consistent with the results obtained with mAbED-C99, 2 h treatment of CHO/APPwt cells with mAbED-C99 F(ab′)2 fragment dose-dependently reduced sAPPα expression (Figure 2a, top panel) and its levels (Figure 2b, upper panel), while leaving secreted Aβ1–40/42 abundances (Figure 2a, middle panel) and their levels (Figure 2b, lower panel) unaltered at the doses examined. Treatment with mAbED-C99 F(ab′)2 fragment also slightly reduced secreted β-CTF levels (Figure 2a, lower panel), further supporting that specific binding of mAbED-C99 to the extracellular domain of β-CTF reduces β-cleavage of human wild-type APP. Moreover, mAbED-C99 F(ab′)2 fragment also dramatically enhanced cell surface β-CTF levels (Figure 2c, right panel) as well as WB band density ratio of cell surface β-CTF to cell surface x-CTF in CHO/APPwt cells (Figure 2d). The increased level of β-CTF is further confirmed using mAbBAM10 that is specific for β-CTF but not x-CTF (Figure 2c, middle panels).

Most importantly, the monoclonal anti-Aβ-actin antibody clearly detected not only β-actin but also IgG1 heavy and light chains in the whole mAbED-C99-treated condition. In contrast, we only observed IgG1 light chain in the mAbED-C99 F(ab′)2 fragment-treated condition, confirming the purity of the prepared F(ab′)2 fragment. We did not observe IgG1 heavy and light chains in the IgG1-treated condition, suggesting that both mAbED-C99 F(ab′)2 fragment and whole mAbED-C99 can specifically bind to membrane-associated full-length human wild-type APP, whereas control IgG1 cannot (Figure 2c, lower panels).

Specific binding of mAbED-C99 or its F(ab′)2 fragment directly inhibits ADAM10-mediated β-cleavage of human wild-type APP. We hypothesized that the decreased sAPPα and β-CTF levels elicited by mAbED-C99 or its F(ab′)2 fragment might be because of the direct inhibition of ADAM10-mediated β-cleavage of human wild-type APP. As expected, compared with IgG1-treated control, WB analysis showed that both whole mAbED-C99 and F(ab′)2 fragment significantly reduced WB band density ratio of β-CTF to full-length human wild-type APP, after incubation of full-length recombinant human wild-type APP protein with active ADAM10 in a cell-free system (Figure 3). These results clearly suggest that the binding of mAbED-C99 or its F(ab′)2 fragment to ED-C99 blocks ADAM10 from proteolysing human wild-type APP at β-cleavage sites.

Specific binding of mAbED-C99 reduces APP endocytosis while increasing cell surface β-CTF. After reaching cell surface via secretory pathway, the matured human wild-type APP molecules could either be predominantly proteolyzed by β-secretases or rapidly endocytosed into the early endosomes through endocytosis pathway and then metabolized by β- and γ-secretases to generate Aβ.12,13 As mAbED-C99 did not alter levels of Aβ, we hypothesized that specific binding to ED-C99 may reduce APP endocytosis. In order to confirm whether mAbED-C99 binding to APPβ672–699 region could modulate human wild-type APP endocytosis, we further assessed the impacts of mAbED-C99 on full-length APP accumulations on the cell surface using the cell surface biotinylation technique.14 As compared with IgG1 isotype control, WB analysis of biotinylated proteins revealed that 2 h treatment of CHO/APPwt cells with mAbED-C99 markedly increased cell surface full-length APP level (Figure 4a, right panel) as well as significantly enhanced WB band density ratio of cell surface full-length APP to pan cadherin (Figure 4b, right panel). In parallel with these findings in CHO/APPwt cells with mAbED-C99 also significantly increased cell surface full-length APP level (Figure 4c, right panel) as well as significantly increased WB band density ratio of cell surface full-length APP to pan cadherin (Figure 4d, right panel). The accumulation of cell surface APP was confirmed by flow cytometry that revealed that 2 h treatment of CHO/APPwt cells with mAbED-C99 led to a significant higher percentage of APP-positive cells when compared with IgG1 isotype control (Figures 4e and f). These results suggest that specific antibody binding to ED-C99 indeed reduces APP endocytosis. Interestingly, mAbED-C99 also dramatically enhanced cell surface β-CTF levels (Figures 4a and c, right panels) as well as significantly increased WB band density ratio of β-CTF to pan cadherin (Figures 4b and d, left panels) in both CHO/APPwt cells and primary cultured cortical cells derived from TgAPPwt mice with mAbED-C99, which further confirmed by mAbBAM10, an antibody specifically recognizing β-CTF but not γ-CTF (Supplementary Figure S2). Taken together, these findings suggest that the specific bindings of mAbED-C99 to APPβ672–699 region inhibits human wild-type APP β-cleavage, while also blocking human wild-type APP endocytosis and promoting its β-cleavage and/or cell surface accumulation of β-CTF.
Specific mAbED-C99 binding enhances the colocalization of human wild-type APP with cholesterol. As growing evidence have suggested that cholesterol is of particular importance in regulating APP processing, favoring β-cleavage and amyloidogenic processing, we further determined colocalization of human wild-type APP with cholesterol in CHO/APP<sub>wt</sub> cells following 2 h mAbED-C99 treatment. Although 2 h treatment with mAbED-C99 increased the cholesterol level on both cellular and subcellular plasma membranes (as indicated by dispersed filipin staining, Figure 5a), this enhanced human wild-type APP with cholesterol colocalization was primarily observed on cell surface but rarely in intracellular compartments (Supplementary Figure S3), indicating that the mAbED-C99-induced human APP binding on APP<sub>672–699</sub> promotes APP β-cleavage. 

Cell Death and Disease
wild-type APP with cholesterol colocalization is cell surface specific. In addition, merged image of cholesterol staining with fillipin (green) and rabbit anti-APP-C-terminal antibody labeling with anti-IgG-594 (red) revealed a higher colocalization of human wild-type APP with cholesterol in mAbED-C99-treated CHO/APPwt cells compared with IgG1 isotype-treated control cells (Figure 5b). This cell surface colocalization of APP with cholesterol may further reduce APP α-CITF and favor β-CITF and cell surface β-CTF accumulation.

**LRP1-CT overexpression reverses mAbED-C99-mediated cell surface accumulation of β-CTF.** APP endocytosis is known to be mediated by binding to the low-density lipoprotein receptor-related protein-1 (LRP1). To further confirm that mAbED-C99 reduces APP endocytosis while enhancing cell membrane β-CTF accumulation, CHO/APPwt cells overexpressing the cytoplasmic tail of LRP1 (LRP1-CT) were exposed with mAbED-C99 for 2 h. Both WB and enzyme-linked immunosorbent assay (ELISA) analyses clearly suggested that although 2 h treatment of CHO/APPwt cells with mAbED-C99 promoted cell surface β-CTF accumulation as compared with IgG1 treatment (Figure 6a, upper panel, lanes 1 and 2), this change was dramatically reversed by overexpressed LRP1-CT (Figure 6a, upper panel, lanes 3 and 4). Surprisingly, LRP1-CT overexpression also significantly but partially reversed the mAbED-C99-mediated decrease of the secreted sAPPα abundances in the conditioned media (Figure 6b, upper panel, lanes 3 and 4) as well as its levels (Figure 6b, middle panel). Most interestingly, the secreted Aβ1-40/42 levels in the conditioned media were significantly elevated in CHO/APPwt/LRP1-CT cells following the reversed inhibition of APP endocytosis (Figure 6b, lower panel).

**mAbED-C99 promotes β-CITF cleavage of human wild-type APP in vivo.** Eight-month-old TgAPPwt female mice were treated with mAbED-C99 or isotype IgG1 as negative control via intracerebroventricular (i.c.v.) injection. At 24 h after treatment, we found that WB band ratio of membrane-bound β-CTF to total APP in the mAbED-C99-treated group was significantly higher than that in the IgG1-treated control group (Figure 7a, middle panels, and Figure 7b, upper panel). Most interestingly, consistent with our in vitro data, mAbED-C99 did not alter the levels of Aβ species (Figure 7a, lower panels). Aβ ELISA analysis of brain homogenates also confirmed that Aβ1-40/42 species were not significantly changed in the mAbED-C99-treated group compared with IgG1 control group (Figure 7b, lower panel). In addition, 8-month-old 5 × FAD female transgenic mice (Tg6799 line) were treated with mAbED-C99 or isotype IgG1 as negative control via i.c.v. injection. At 24 h after treatment, we found that immunohistochemical staining also disclosed comparable amount of β-amyloid plaques in retrosplenial cortex (RSC), entorhinal cortex (EC), and hippocampus (H) regions of 5 × FAD transgenic mouse brains (Supplementary Figure S4).

**Discussion**

Mutations in APP gene cause early onset of autosomal-dominant AD. Relative to their wild-type homologs, the English (APP677 H→R) and Tottori (APP678 D→N) substitutions accelerate the kinetics of Aβ secondary structure change from statistical coil → α/β → β and produce oligomer size distributions skewed to higher order that are more toxic to cultured neuronal cells than wild-type oligomers. The Icelandic APP672 mutation (A→V) affects APP processing, resulting in enhanced Aβ production (quantity) and formation of amyloid fibrils in vitro (quality). In contrast, alternative APP675 mutation (A→T) results in an ~40% reduction in the formation of amyloidogenic peptides and therefore protects against AD and cognitive decline in the elderly. Thus, these pathogenic mutations located in the APP672 to APP699 region of APP ectodomain (also named ED-C99) encompassing APP α- and β-CITF cleavage sites can alter β-CITF and Aβ1-40/42-related AD pathology. However, unlike autosomal-dominant FAD, SAD patients generally lack mutations of APP gene. Therefore, the mechanisms underlying pathogenesis of SAD
are still far from clarification. Here, we hypothesized that modifications (either physical or functional interactions) of regions close to APP \( \alpha \)- and \( \beta \)-cleavage sites by either endogenous or exogenous molecules may yield similar impacts on human wild-type APP processing to what is observed with APP mutations.
Cell surface α-cleavage is the predominant processing pathway for human wild-type APP. In the present study, we found that the specific binding of the APP ED-C99 region with mAbED-C99 dose-dependently blocks α - but promotes β -cleavage of human wild-type APP (Figure 1). These effects were further confirmed by another specific antibody, 4G8, recognizing the ED-C99 domain (with epitope at APP688-695, data not shown). In contrast, N-terminal APP antibody 22C11 (epitope of APP66-81) or sAPPα-specific antibody mAb2B3 (epitope of APP672-688, absence of affinity to full-length APP) showed no effect on human wild-type APP processing. This lack of impact of mAb22C11 and mAb2B3 further indicates that the effect of mAbED-C99 on human wild-type APP processing is region specific. In addition, these modulations on human wild-type APP α/β-cleavage induced by mAbED-C99 can be recapitulated by using the F(ab′)2 fragment of mAbED-C99 (Figure 2). As F(ab′)2 lacks the nonspecific Fc binding subunit, these results confirm that mAbED-C99-induced modification of APP processing is ED-C99 region specific. Our present study further reveals that this α-cleavage inhibiting activity is due, in part, to the direct physical blocking of ADAM10 (Figure 3).

Figure 5  Colocalization of human wild-type APP with cholesterol in CHO/APPwt cells after mAb ED-C99 treatment. (a) CHO/APPwt cells were plated to 8-well slide chamber and then, after overnight incubation, the cells were treated with mAbED-C99 or IgG1 isotype control for 2 h. These cells were stained by Filipin in strict accordance with the manufacturer’s instructions of the cholesterol assay kit. (b) After Filipin staining and washing, some of these cells were permeabilized with 0.05% Triton X-100 for 5 min, washed, and stained with rabbit anti-APP-C-terminal antibody overnight at 4°C. Alexa Fluor 594 Donkey anti-rabbit IgG was used to detect APP signals. Confocal images were taken by Olympus Fluoview FV1000 laser scanning confocal microscope (Tokyo, Japan)

Figure 4  Cell surface β-CTF and full-length APP are increased following treatment with mAbED-C99. (a) CHO/APPwt cells in 24-well plates (5 × 10^5/well) were treated with mAbED-C99 or IgG1 isotype control at 1.25 μg/ml for 2 h, washed three times with PBS-CM, and then cell lysate portions of these cells were directly subjected to WB analysis using pAb751/770 (left panels). The remaining cells were biotinylated with Sulfo-NHS-LC-Biotin, quenched with NH4Cl-PBS-CM, lysed, and immunoprecipitated (IP) using Neutravidin beads. The intracellular proteins obtained by IP/Neutravidin depletion (middle panels) and the cell surface (cell surf) proteins obtained by IP/Neutravidin precipitation (right panels) were subjected to WB analysis using pAb751/770. As shown below each panel, as an internal control, β-actin was analyzed for cell lysates and intracellular (intra) proteins and pan cadherin was analyzed for total cell surface proteins. (b) For WB quantitative analysis, band density ratios of cell surface β-CTF or full-length APP to pan cadherin were analyzed and presented as mean ± S.D. (**P < 0.01, ***P < 0.001). The WB data are representative of three independent experiments with similar results. (c) Primary neuronal cells were cultured from cortical tissues of 1-day-old TgAPP wt mouse pups and replated in 24-well plates at 2 × 10^5/well overnight. These primary neuronal cells were treated with mAb ED-C99 or IgG1 isotype control at 1.25 μg/ml for 2 h, washed three times with PBS-CM and then cell lysates were directly subjected to WB analysis using pAb751/770 (left panels). The remaining cells were biotinylated, immunoprecipitated with Neutravidin beads, and then subjected to isolation of intracellular and cell surface proteins. The intracellular proteins obtained by IP/Neutravidin depletion (middle panels) and the cell surface (cell surf) proteins obtained by IP/Neutravidin isolation (right panels) were subjected to WB analysis using pAb751/770. (d) For WB quantitative analysis, band density ratios of cell surface β-CTF or holo APP to pan cadherin were analyzed and presented as mean ± S.D. (**P < 0.01, ***P < 0.001). These WB data are representative of two independent experiments with similar results. (e) Flow cytometry analysis of cell surface APP utilizing a rabbit anti-N-terminal APP antibody. (f) Percentage of full-length APP-positive cells are presented as mean ± S.D. (***P < 0.001)
Figure 6  Overexpressing LRP1-CT markedly restores human wild-type APP endocytosis in CHO/APP<sub>wt</sub>/LRP1-CT cells. CHO/APP<sub>wt</sub>/LRP1-CT or CHO/APP<sub>wt</sub> cells were treated with mAb ED-C99 for 2 h. (a) Full-length APP and α/β-CTFs were examined by WB analysis. (b) The secreted sAPP<sub>α</sub> and Aβ<sub>1–40/42</sub> levels in conditioned media were measured by WB analysis and ELISA. The band ratio of β-CTF to α-CTF and sAPP<sub>α</sub> ELISA results (ng of sAPP<sub>α</sub> per mg of total proteins) are presented as mean ± S.D. These data are representative of three independent experiments with similar results (*P < 0.05).

Figure 7  The mAb ED-C99 promotes APP β-secretase processing in vivo. TgAPP<sub>40/40</sub> female mice at 8 months of age were treated with mAb ED-C99 or control IgG<sub>1</sub> at 5 μg/mouse by intracerebroventricular (i.c.v.) injection and killed 24 h after the treatment (n = 6). (a) Cytosolic- (left panels) and membrane-associated proteins (right panels) prepared from mouse brain homogenates were subjected to WB analysis for APP processing. (b) For WB quantitative analysis, band density ratio of membrane-associated β-CTF to membrane-associated total APP was analyzed (upper panel). Aβ<sub>40/42</sub> was also analyzed by ELISA (lower panel, n = 6). The results are presented as pg of Aβ<sub>40/42</sub> per mg of total intercellular proteins (mean ± S.D.; ***P < 0.001).
Previous studies have suggested that intracellular trafficking is pivotal for human wild-type APP proteolysis. In contrast, non-amyloidogenic processing occurs mainly at the cell surface, where z-secretases are present. Amyloidogenic processing involves transit through the endocytic organelles, where APP encounters z- and y-secretases. In our present study, mAbED-C99 enhanced cell surface APP accumulation, which was confirmed by both WB and flow cytometry analyses, indicating that ED-C99 binding reduces the APP endocytic pathway. Blockage of human wild-type APP z-secretase by mAbED-C99 may consequently lead to an elevated y-secretase that has been confirmed by dramatically elevated z-CTF. This suggests that ED-C99 may bind and trap APP in the membrane (or endosomes in the process of fusing with the plasma membrane), making the proteolytic sites for ADAM10 inaccessible, and thus shifting the proteolysis of APP in the plasma membrane or endosomes to y-secretase cleavage.

This proposed blockage of human wild-type APP z-secretase by mAbED-C99 may consequently lead to an elevated y-secretase that has been confirmed by the dramatically increased y-CTF levels on the cell surface (Figure 4). Our findings suggest that this elevated y-secretase can be because of the cell surface APP with cholesterol colocalization (Figure 5). Indeed, growing evidence has demonstrated that cholesterol is of particular importance in regulating z- and y-secretase cleavage of APP. In contrast to y-secretase, previous studies have indicated that y-secretase cleavage of C99 appears to occur primarily in rafts located in the endosomes. Cholesterol can bind to APP, an N-loop structure at the end of the ED-C99 region, thereby inhibiting z-secretase while favoring y-secretase of APP. In contrast to y-secretase, previous studies have indicated that y-secretase cleavage of C99 appears to occur primarily in rafts located in the endosomes. In contrast to the cell surface APP with cholesterol colocalization (Figure 5). Indeed, growing evidence has demonstrated that cholesterol is of particular importance in regulating z- and y-secretases.

In our present study, mAbED-C99 treatment also yielded no significant changes of Aβ/total APP (Figure 7b). Similar to our in vitro study, mAbED-C99 treatment also yielded no significant changes of Aβ production compared with the IgG control. Although the amyloid cascade hypothesis is potentially viable in cases of genetic mutation-caused autosomal-dominant fAD, accumulating evidence suggests that it may not apply in the vast majority of patients with late-onset sAD lacking mutations of APP/presenilin genes.

These works suggest several possibilities in terms of defining a possible etiology and treatment target for both fAD and sAD. First, Aβ-related plaques is one relatively common finding in the non-demented elderly. In fact, recent studies have demonstrated that accumulation of APP y-CTF may have direct deleterious effects on cognitive function. For example, inhibition of y-site amyloid cleaving enzyme (BACE) rescued synaptic/memory deficits in a mouse model of familial Danish dementia. However, y-secretase inhibition worsened memory deficits in these mice that correlated with increased levels of APP y-secretase. In another report, prolonged (8 days) treatment with y-secretase inhibitors (GSIs) produced no positive effects on memory deficits of older Swedish APP transgenic mice, but induced cognitive deficits in young Swedish APP transgenic mice or wild-type mice. Indeed, a recent phase III clinical trial with the GSI Semagacestat was halted because of worsened clinical measures of cognition and the ability to perform activities of daily living. These results suggest that y-CTF rather than Aβ may be more directly responsible for causing cognitive impairment associated with AD and that GSIs may worsen cognitive impairment by enhancing the accumulation of y-CTF. Our results also point to a need to identify, epidemiologically, the presence or absence of APP y-secretase-like antibodies or proteins that may increase with age and correlate with onset of AD-like signs and symptoms. In addition, this work suggests that such an antibody or protein could reduce y-secretase function and thus generation of sAPPβ, a peptide that is neuroprotective.

In summary, the present study verifies our hypothesis that ED-C99 region (APP 672–699) is critical for human wild-type APP processing. Specific binding of this region will directly inhibit APP y-secretase activity and reduce APP endocytosis, thereby enhancing cell surface y-CTF accumulation. These effects may have deleterious effects of cognition, and this should be further explored in future studies. Modifications (physical or functional interactions) of this region with either exogenous or endogenous molecules will affect human wild-type APP processing, potentially enhancing or ameliorating the development of AD (Supplementary Figure S5).

Materials and Methods

Antibodies. Sterile and low-endotoxin antibodies were used, including anti-C-terminal human sAPPβ-specific antibody 2B3 (mAb2B3; IBL, Minneapolis, MN, USA), APP C-terminal antibody 22C11 (m22C11; Roche, Basel, Switzerland), specific monoclonal ED-C99 antibody (mAbED-C99, 6E10; Covance, Emeryville, CA, USA), APP C-terminal antibody pAb751/770 (EMD Biosciences, La Jolla, CA, USA), specific monoclonal antibody BAM10 (Sigma-Aldrich, St Louis, MO, USA), β-actin antibody (Sigma-Aldrich), and anti-pan cadiherin antibody (AbCam, Cambridge, MA, USA). The sAPPβ-specific 2B3 antibody was further characterized in our in vitro and in vivo systems, indicating that this antibody recognizes neither Aβ nor full-length APP. The mAbED-C99 Fab(ab′)2 fragment was generated using F(ab′)2.
preparation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

**Cell culture and treatment.** CHO cells engineered to express wild-type human APP (CHO/APP<sub>wt</sub>) were kindly provided by Dr. Stanie Hahn and Dr. Sascha Weggen (University of Heinrich Heine, Düsseldorf, Germany). Plasmid PHCHX-LRP1-CT-G1 was a generous gift from Dr. David Kang (University of South Florida, Tampa, FL, USA). LRP1-CT was subcloned to PCDNA vector by PCR and restriction enzyme HindIII and Not digestion. The primers were used as follows: forward 5’-AGCTGGTTGATGGAATTGACT-3’ and reverse 5’-ATGC GGCCTGCTAGCAACGGTTCCTATC-3’. Stable cell lines were generated by transfection of PCDNA-LRP1-CT into CHO/APP<sub>wt</sub> cells and single colony was picked up after G418 administration. These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, 100 μU/ml penicillin/streptomycin. For TgAPP<sub>wt</sub> mice, mouse-derived cortical neurons, cerebral cortices isolated from 1-day-old TgAPP<sub>wt</sub> mice were mechanically dissociated in trypsin (0.25%) individually after incubation for 15 min at 37°C. Cells were collected after centrifugation at 1200 × g, suspended in DMEM supplemented with 10% fetal calf serum, 1% horse serum, uridine (33.6 μg/ml), Sigma-Aldrich), and fluorodeoxyuridine (13.6 μg/ml, Sigma-Aldrich) and seeded in 24-well collagen-coated culture plates at 2.5 × 10<sup>5</sup> cells per well. After reaching confluence (~70–80%), cells were treated with mAbED-C99 at 0–1.25 μg/ml for 2 h. In additional experiments, cells were treated with mAb22C11 or mAb2B3 or mAbED-C99 F(ab'<sub>1</sub>) fragment at 1.25 μg/ml.

**Transgenic APP<sub>wt</sub> mice and i.c.v. injection.** Transgenic wild-type B6.Cg-Tg (PDGFβ-APP) S614S17 strain (TgAPP<sub>wt</sub>) female mice and 5 × FAD transgenic female mice (Tg6799 line) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed and maintained in the Animal Facility of College of Medicine at University of South Florida. At 8 months of age, both mice were anesthetized using isoflurane (chamber induction at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. i.c.v. injection of 200 ng of mAbED-C99 and isotype control IgG<sub>1</sub> were dissolved in sterile distilled water at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. At 8 months of age, both mice were anesthetized using isoflurane (chamber induction at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. At 8 months of age, both mice were anesthetized using isoflurane (chamber induction at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. At 8 months of age, both mice were anesthetized using isoflurane (chamber induction at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min. After reflexes were blocked, the animal was placed under a binocular stereomicroscope and a 2-mm incision made on the left lateral ventricle with a microsyringe at a rate of 1 μl/min.
12. Thinaikaran G, Koo EH. Amyloid precursor protein trafficking, processing, and function. J Biol Chem 2008; 283: 29615–29619.
13. Choy RW, Cheng Z, Schekman R. Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. Proc Natl Acad Sci USA 2012; 109: E2077–E2082.
14. Walz T, Heilig C, Schweitzer A, Niedera N, Jaeger S, Martin AM et al. LRP1 modulates APP trafficking along early compartments of the secretory pathway. Neurobiol Dis 2008; 31: 188–197.
15. Marquard C, Devauges V, Cossec J, Liott G, Lécart S, Saoud F et al. Local cholesterol increase triggers amyloid precursor protein-Bace1 clustering in lipid rafts and rapid endocytosis. FASEB J 2011; 25: 1296–1305.
16. Kojro E, Gimpl G, Lammich S, Marz W, Fahrenholz F. Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the γ-secretase ADAM. Proc Natl Acad Sci USA 2001; 98: 5815–5820.
17. Ehehalt R, Keller P, Haass C, Thiele C, Simons K. Amyloidogenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts. J Cell Biol 2003; 160: 113–123.
18. De Jonghe C, Eseleens C, Kumar-Singh S, Craessaerts K, Serneels S, Checler F et al. Pathogenic APP mutations near the γ-secretase cleavage site differentially affect APP C-terminal fragment stability. Hum Mol Genet 2001; 10: 1665–1671.
19. Brouwers N, Siegers K, Van Broeckhoven C. Molecular genetics of Alzheimer’s disease: an update. Ann Med 2008; 40: 562–583.
20. Wilquet V, De Strooper B. Amyloid-β precursor protein processing in neurodegeneration. Curr Opin Neurobiol 2004; 14: 582–589.
21. Ono K, Condron MM, Tlelew DB. Effects of the English (H6R) and Tottori (D7N) familial Alzheimer disease mutations on amyloid β-protein assembly and toxicity. J Biol Chem 2010; 285: 23186–23197.
22. Guardia-Laguarta C, Atsalis T, Strainberg S, Snaedal J, Jonsson PV, Bjornsson S et al. Modulation of γ- and β-secretase activity and amyloid-β generation by presenilin-1 and -2. J Neurochem 2011; 113: 188–197.
23. Guardia-Laguarta C, Coma M, Pera M, Clarimon J, Snaedal J, Bjornsson S et al. Differential effects of γ- and β-secretase modulators regardless of their potency and structural similarity. J Neurochem 2011; 116: 385–395.
24. Mattson MP, Kuribara K. Signaling events regulating the neurodevelopmental triad. Glutamate and secreted forms of amyloid-β precursor protein as examples. Perspect Dev Neurosci 1998; 5: 337–352.
25. Mattson MP, Gary DS, Chan SL, Duan W. Perturbed endoplasmic reticulum function, synaptic apoptosis and the pathogenesis of Alzheimer’s disease. Biochem Soc Symp 2001; 67: 151–162.
26. Hahn S, Bruning T, Ness J, Czirr E, Baches S, Gijsen H et al. Presenilin-1 but not amyloid precursor protein mutations present in mouse models of Alzheimer’s disease attenuate the response of cultured cells to γ-secretase modulators regardless of their potency and structure. J Neurochem 2011; 116: 385–395.
27. Deng J, Hou H, Guinta B, Mori T, Wang YJ, Fernandez F et al. Autoreactive-Aβ antibodies promote APP γ-secretase processing. J Neurochem 2012; 120: 732–740.
28. Rezai-Zadeh K, Shytie D, Sun N, Mori T, Hou H, Jeannot D et al. Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. J Neurosci 2005; 25: 8807–8814.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)