

## Cell-type dependent modulation of Notch signaling by the amyloid precursor protein

Sun Young Oh, Ci-Di Chen and Carmela R. Abraham

Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts, USA

### Abstract

The amyloid precursor protein is a ubiquitously expressed transmembrane protein that has been long implicated in the pathogenesis of Alzheimer's disease but its normal biological function has remained elusive despite extensive effort. We have previously reported the identification of Notch2 as an amyloid precursor protein interacting protein in E18 rat neurons. Here, we sought to reveal the physiologic consequences of this interaction. We report a functional relationship between amyloid precursor protein and Notch1, which does not affect Delta ligand binding. First, we observed interactions between the amyloid precursor protein and Notch in mouse embryonic stem cells lacking both presenilin 1 and presenilin 2, the active proteolytic components of the gamma-secretase complex, suggesting that these two transmembrane proteins can

interact in the absence of presenilin. Next, we demonstrated that the amyloid precursor protein affects Notch signaling by using Notch-dependent luciferase assays in two cell lines, the human embryonic kidney 293 and the monkey kidney, COS7. We found that the amyloid precursor protein exerts opposing effects on Notch signaling in human embryonic kidney 293 vs. COS7 cells. Finally, we show that more Notch Intracellular Domain is found in the nucleus in the presence of exogenous amyloid precursor protein or its intracellular domain, suggesting the mechanism by which the amyloid precursor protein affects Notch signaling in certain cells. Our results provide evidence of potentially important communications between the amyloid precursor protein and Notch.

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It has been known for over two decades that the amyloid precursor protein (APP) is a key protein in the pathogenesis of Alzheimer's disease (AD) because of its sequential proteolytic cleavages by the  $\beta$  and  $\gamma$ -secretases to generate the cytotoxic and synaptotoxic amyloid beta (A $\beta$ ) peptides (Selkoe 1999; Hardy and Selkoe 2002). Furthermore, mutations in APP lead to early onset, dominantly inherited AD. Despite the well-acknowledged role of APP in AD pathology, the current understanding of the normal physiological functions of APP remains to be further elucidated. Efforts from many laboratories have provided evidence for the role of APP in a variety of signaling pathways through the interaction of APP with other molecules. These include cytosolic adaptor proteins such as Fe65 (Cao and Sudhof 2001; Von Rotz *et al.* 2004) and Mint/X11 $\alpha$  (Butz *et al.* 1998; Sastre *et al.* 1998; Setou *et al.* 2000; Biederer *et al.* 2002). APP also binds to the cell surface receptor Nogo-66 (Park *et al.* 2006) and sortilin-related receptor with A-type repeats (SORLA) (Andersen *et al.* 2005; Offe *et al.* 2006). Interestingly, polymorphisms in SORLA have been shown to be associated with late onset AD (Rogaeva *et al.* 2007). A recent study reported the interaction between APP and transient axonal glycoprotein 1, a member of F3/contactin

family associated with the negative modulation of neurogenesis in embryonic mouse brain (Ma *et al.* 2008). Finally, F-spondin is a neuronally secreted glycoprotein, which also binds to the extracellular domain of both APP and amyloid precursor protein-like protein (APLP) 2 (Ho and Sudhof

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Author correspondence and reprint requests to Carmela R. Abraham, Department of Biochemistry, Boston University School of Medicine, 72 East Concord Street, K620, Boston, MA, USA.

E-mail: cabraham@bu.edu

**Abbreviations used:** AD, Alzheimer's disease; AICD, APP intracellular domain; APLP, amyloid precursor protein-like protein; APP, amyloid precursor protein; BiFC, bimolecular fluorescence complementation; CBF1, C promoter-binding factor 1; CHO, Chinese Hamster Ovary; co-IP, co-immunoprecipitation; D11Fc, Delta1-Fc; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; FLN, full-length Notch; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; HA, hemagglutinin; HEK293, human embryonic kidney 293 cell; NCT, nicastrin; NICD, Notch intracellular domain; PBS, phosphate buffered saline; PS, presenilin; PS1CTF, presenilin 1 C-terminal fragment; PS1NTF, presenilin 1 N-terminal fragment; PSdKO, presenilin double knockout (PS1 $-/-$ /PS2 $-/-$ ); qPCR, quantitative real-time PCR; SORLA, sortilin-related receptor with A-type repeats; YFP, yellow fluorescence protein.

2004). These binding partners affect APP processing when over-expressed in various cell lines.

Recent *in vivo* studies provide compelling evidence for APP's role in neuronal migration and neurite outgrowth. *In utero* electroporation of short hairpin RNA into the developing mouse cortex to knock down APP results in defects in neuronal migration to the cortical plate (Young-Pearse *et al.* 2007), showing the requirement of APP in migration of neuronal precursors. Deletion of APP, APLP1, or APLP2 in neurons induces elongation of neurites. The effect on neurite outgrowth is mediated by secreted APP, which competes with full length APP for binding to integrin  $\beta 1$  (Young-Pearse *et al.* 2008).

In addition to these studies, we, and others identified Notch 2, as a binding partner of APP (Fassa *et al.* 2005; Fischer *et al.* 2005; Oh *et al.* 2005; Chen *et al.* 2006). Notch receptors are type I transmembrane proteins playing a critical role in both vertebrates and invertebrates during embryonic development and throughout the adult life. Notch proteins are evolutionarily conserved and implicated in cell fate determination and various other functions in many organisms (Artavanis-Tsakonas *et al.* 1995, 1999; Kopan and Turner 1996; Weinmaster 1997). Also, elements of Notch signaling similarly show a high degree of conservation across species, further confirming the essential role of Notch biology. Notch receptors and ligands are differentially expressed in adjacent cells, ensuring proper, directional signaling. Upon ligand binding, Notch receptors undergo N-terminal cleavage mediated by a disintegrin and metalloprotease-family of metalloproteases followed by gamma-secretase cleavage, which occurs within the transmembrane region of the Notch receptor. The gamma-secretase processing of Notch releases the Notch intracellular domain (NICD), which then translocates to the nucleus to regulate transcription via interactions with the CSL [C-promoter binding factor 1 (CBF1), suppressor of hairless (Su(H)), lin-12 and glp-1 (Lag-1)] family of DNA binding proteins and the transcriptional co-activator Mastermind. Notch signaling inhibits neurite extension (Berezovska *et al.* 1999; Sestan *et al.* 1999; Redmond *et al.* 2000) whereas the inhibition of Notch signaling promotes neurite extension. The effects of Notch activation were examined by expression of Notch target genes like *Hes1*, in the developing mouse embryos where inhibition of neuronal differentiation was found (Ohtsuka *et al.* 2001; Sakamoto *et al.* 2003). These results suggest that Notch activation is a critical inhibitor supporting the normal differentiation patterning of the CNS.

While it is clear that Notch is critical in development of the CNS, data from a gene expression study show Notch pathway molecules expressed in both the early postnatal and then again in the adult brain providing evidence for the importance of Notch function in the adult CNS (Berezovska *et al.* 1997, 1998; Stump *et al.* 2002). Additionally, several studies report the role of Notch in the adult brain using loss-

of-function approaches. Mice containing heterozygous mutations for either Notch1 or the CSL family of DNA-binding transcription factor, CBF/RBPj, show deficits in spatial learning and memory (Costa *et al.* 2003).

It is important to note that APP and Notch are two substrates of the gamma-secretase complex mediated regulated intramembrane proteolysis (Ebinu and Yankner 2002). In fact, a significant analogy in the proteolytic processing of both APP and Notch can be found during the two sequential cleavages of regulated intramembrane proteolysis. Both receptors undergo an extracellular shedding by a disintegrin and metalloproteases, gamma-secretase, with the subsequent intramembrane cleavage mediated by the gamma-secretase. It is thus intriguing to consider whether APP and Notch are involved in similar or opposing cellular processes.

To expand upon our previous work and attempt to elucidate the functional consequences of an interaction between APP and Notch, we investigated whether APP could modulate Notch signaling in two different mammalian cell lines, human embryonic kidney (HEK) 293 and COS7. Here, we provide evidence that APP differentially modulates Notch signaling. We found that the modulation can be either positive or negative, depending on the cell type examined.

## Materials and methods

### Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate, puromycin, and zeocin were from Invitrogen (Carlsbad, CA, USA). Mouse embryonic stem cells deficient in both presenilin-1 and presenilin-2 were obtained from Dr. B. Yankner at Harvard Medical School (Boston, MA, USA). Leukemia inhibitory factor and 2-mercaptoethanol, hygromycin, and blasticidin were from Sigma (Missouri, CA, USA). The Chinese Hamster Ovary (CHO) cell lines,  $\gamma$ -30 [stably over-expressing human APP751, human PS1, Flag-PEN2 [presenilin enhancer 2 homolog (*C. elegans*)], and Aph1 $\alpha$ 2-hemagglutinin (HA)] and S1 [stably over-expressing human APP, human PS1, Flag-PEN2, Aph1 $\alpha$ 2-HA, and nicastrin-glutathione-S-transferase (NCT-GST)] were obtained from Dr. M. Wolfe at Harvard Medical School (Boston, MA, USA). CBF1-luciferase reporter construct, pGL3JH26 (Hicks *et al.* 2000), containing eight tandem repeats of CBF1 responsive DNA binding site and the pCDNA1-DIIFc encoding the secreted Notch ligand encoding first 467 amino acids of rat Delta 1 fused to the human Fc fragment (DIIFc) were kindly provided by Dr. G. Weinmaster (UCLA). FITC-conjugated goat anti-human Fc antibody (FITC-goat Fc) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Hairpin/enhancer of split 1-specific primers and probe (Hs00172878\_m1) with 6FAM fluorescein, cyclophilin with 6FAM probe, and TaqMan Gene Expression Assay kit were purchased from Applied Biosystems (Foster City, CA, USA). All the plasmids encoding fluorescent fusion proteins used for bimolecular fluorescence complementation (BiFC) experiments were previously described (Oh *et al.* 2005; Chen *et al.* 2006). Other expression vectors used to transfect cells were as

follows: pEF-Bos empty vector, pCDNA3-APP, pBos-full-length Notch (FLN) 1, pBos-NICD, pCDNA3-FLN2, APP/YFP-pCDNA1, APP/YN-pCDNA1, APP/YC-pCDNA1, FLN2/YN-pCDNA1, pHRL-SV40, and pCDNA-C50-Myc (obtained from Dr. B. Yankner).

### Cell culture

All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere. HEK293 and COS7 cells were cultured in DMEM containing 10% FBS and 100 units/mL penicillin and streptomycin. The presenilin (PS) double knockout (PSdKO) cells (Zhang *et al.* 2000) were grown in DMEM supplemented with 15% FBS, 292 µg/mL L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate, 1000 units/mL of leukemia inhibitory factor, and 0.008% 2-mercaptoethanol. Gamma30 CHO cell line over-expressing human PS1, Flag-Pen2, and APh1α2-HA (Kimberly *et al.* 2003) were cultured in DMEM containing 10% FBS, 1% L-glutamine, 1% penicillin and streptomycin, G418 (150 µg/mL), puromycin (2.5 µg/mL), zeocin (250 µg/mL), and hygromycin (250 µg/mL). Another CHO cell line, S1, (Fraering *et al.* 2004) over-expressing human PS1, Flag-Pen2, APh1α2-HA was cultured in DMEM containing 10% FBS, 1% L-glutamine, 1% penicillin and streptomycin, G418 (150 µg/mL), puromycin (2.5 µg/mL), zeocin (250 µg/mL), hygromycin (250 µg/mL), and blasticidin (10 µg/mL).

### Luciferase assays

#### *CBF1-luciferase assay*

The transfection of HEK293 or COS7 cells in 24-well plates was performed using Fugene 6 (Roche, Indianapolis, IN, USA) transfection reagent. Cells were transfected with 35 ng of the pGL3JH26, 1 ng of Renilla luciferase reporter, pHRL-SV40 (Promega, Madison, WI, USA), and other indicated expression vectors. Total amount of DNA for each transfection was adjusted to 350 ng/well by adding a control vector (pEF-Bos). Transfected cells were harvested and analyzed 24–48 h after transfection using the Promega Dual-Luciferase Reporter Assay System. The reporter activity was measured using the DR Ready-Luminoscan Ascent (Thermo Electron Informatics, Philadelphia, PA, USA). The data represent the average results from at least three different experiments, each performed in triplicate. Data from the luciferase activity was first normalized to the control Renilla luciferase, pHRL-SV, activity followed by normalization against the control samples (transfected with empty vector).

### Soluble Notch ligand Delta1-Fc binding assay

#### *Preparation of conditioned media and ligand clustering*

The cDNA construct encoding the secreted Notch ligand Delta (Redmond *et al.* 2000) was expressed in COS7 cells by transient transfection using Lipofectamine and Plus Reagent (Invitrogen). Conditioned media (CM) containing the secreted Delta1-Fc (D11Fc) were collected from COS7 cells transfected with 10 µg of D11Fc in 10 cm plates following 48 h post-transfection. The soluble ligands from conditioned media were clustered at 4°C with FITC-goat Fc (1.8 µg/mL) in blocking media for 30 min as described previously (Van Gassen *et al.* 2000). The solution containing clustered ligands was diluted three-fold (0.6 µg/mL final concentration) and added to cells for 30 min at 37°C for ligand binding.

#### *Preparation of cell lysates after ligand binding*

COS7 cells were plated in 6-well plates and transfected with APP, FLN1, or FLN2, co-transfected with APP and FLN1 or APP and FLN2. The total amount of DNA in each well was held constant by transfection of empty vector. Forty-eight hours post-transfection; cells were incubated for 45 min at 37°C in blocking media containing (DMEM containing 10% goat serum and 1% bovine serum albumin). Following the blocking, cells were incubated with the pre-clustered soluble ligand D11Fc for 30 min at 37°C for binding to receptors. After binding, cells were washed with 1× phosphate buffered saline (PBS) and removed by scraping. The cell lysates were collected in 1× PBS and analyzed by flow cytometry.

#### *Flow cytometry*

Forty-eight hours after transfection, cells were washed with PBS, scraped, and suspended in 1 mL PBS. The flow cytometry analysis of the single-cell suspensions was performed using a FACScan (BD Biosciences, San Jose, CA, USA). A minimum of 10 000 events was acquired on a gate including viable cells.

### Real-time PCR

Hairy/enhancer of split 1-specific primers and probe (Hs00172878\_m1) were purchased from Applied Biosystems. Cyclophilin with 6FAM probe was used as a reference/endogenous control gene. COS7 cells in 6-well plates were transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen) transfection reagents. The RNA samples were extracted from the transfected COS7 cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The reverse transcription and amplification steps were performed together using Taqman One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Each reaction contained 2 ng of total RNA as a starting material for the reverse transcription and following amplification in a total volume of 10 µL. Reactions were performed using an ABI PRISM 7900HT PCR machine.

### Bimolecular fluorescence complementation

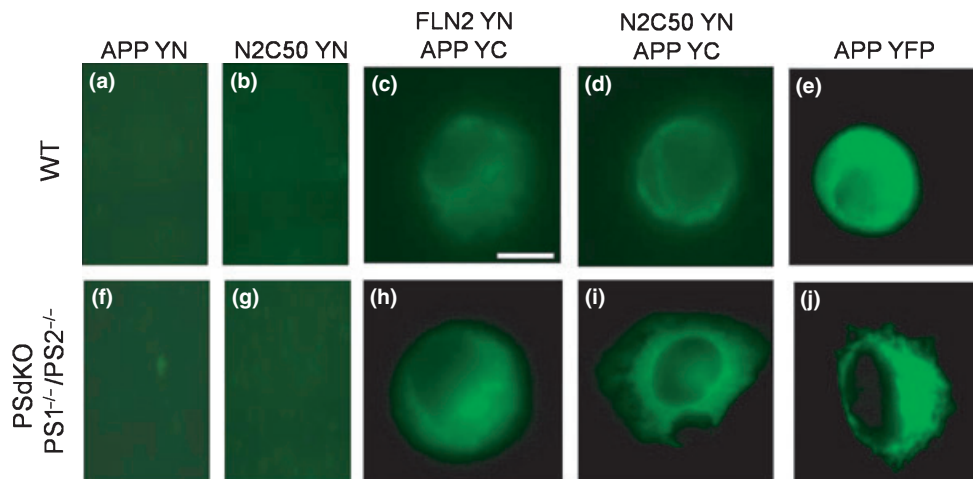
Wild-type and PSdKO (deficient in both PS1 and PS2) mouse embryonic stem cells were transfected in Lab-Tek II 8-chambered cover glass (Nalgene Nunc International, Rochester, NY, USA) using Lipofectamine 2000 (Invitrogen) with the indicated fusion constructs containing the fragments of yellow fluorescence protein (YFP). Twenty four to forty-eight hours post-transfection, cells were washed twice with PBS and the fluorescence was observed in living cells in PBS using a Zeiss Axiovert 200 M microscope with cyan fluorescent protein and YFP filters.

### Nuclear fractionation

COS7 cells were transfected in 6-well plates using Lipofectamine 2000 (Invitrogen) with the indicated plasmids. Cells were harvested 48 h after transfection. Nuclear fraction was prepared using NE-PER Nuclear and Cytosolic Extraction Kit (Pierce, Rockford, IL, USA) following the manufacturer's protocol. Histone H1 was used as a nuclear marker to normalize and quantify nuclear NICD levels.

### Western blotting and antibodies

HEK293 and COS7 cell lysates were analyzed by running on 4–12% Bis-Tris Nu-PAGE gels (Invitrogen) and transferred to



**Fig. 1** APP and Notch interaction does not require presenilin/gamma secretase complex. Embryonic fibroblasts from wild-type (WT) or presenilin double knock-out (PSdKO) mice were transfected with the fusion fluorescent proteins described for bimolecular fluorescent complementation (BiFC). Live cells were visualized following 24–48 h post-transfection. The single transfections with plasmids encoding

only the N-terminal half of the yellow fluorescent protein, APP-YN (a, f) or N2C50-YN (b, g), served as a negative control. Cells transfected with APP-YFP served as a positive control (e, j). BiFC signal was detected in both WT and PSdKO cells co-transfected with FLN2-YN and APP-YC (c, h) or N2C50-YN and APP-YC (d, i).

polyvinylidene fluoride membranes for western blotting with antibodies against the N-terminal fragment of presenilin 1 (PS1NTF, MAB1563, Chemicon/Millipore, Billerica, MA, USA), C-terminal fragment of presenilin 1 (PS1CTF, 4627, gift from Dr. Michael Wolfe), Nicastrin (NCT, Sigma), and APH-1 (Affinity BioReagents, Cambridge, MA, USA). Although the specificity of anti-PS1NTF (amino acids 21–80 of human PS1) to monkey PS1 (as in COS7 cells) has not been tested, it is very likely that this antibody will also recognize PS1 in COS7 cell line as the residues 21–80 are 100% identical in these two species. The specificity of the anti-PS1CTF (Walter *et al.* 1996) in COS7 cells also has not been tested, however the last 10 amino acids at the C-terminus of PS, against which the antibody was produced, are identical in both human and monkey. For western analysis of the NICD, COS7 cell lysates were run on 4–20% Tris-Glycine gels and probed with NICD antibody (Val 1744, Cell Signaling Technology, Danvers, MA, USA). A mouse monoclonal antibody to human Histone H1 (sc-8030) was from Santa Cruz Biotechnology, Santa Cruz, CA, USA and the mouse anti-beta-tubulin antibody was from Zymed Laboratories Inc., South San Francisco, CA, USA. The mouse monoclonal antibody (9E10) to c-Myc was purchased from Abcam (Cambridge, MA, USA).

## Results

### APP and Notch interact in presenilin double knockout mouse embryonic fibroblasts

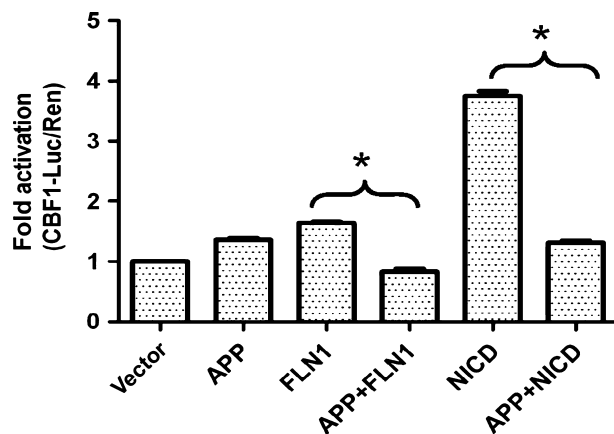
As PS contains the active site of gamma-secretase complex (Esler *et al.* 2002), the absence of PS would prevent the formation of the active enzymatic complex. In order to determine whether APP and Notch interact in mouse embryonic fibroblasts lacking both PS1 and PS2 (PS1<sup>−/−</sup>/

PS2<sup>−/−</sup>; PSdKO) we performed the BiFC assay (Kerppola 2008) to visualize APP and Notch interaction in live cells. We detected similar intensity of fluorescence complementation resulting from APP and Notch heterodimer interaction both in wild-type (Fig. 1c and d) and in PSdKO cells (Fig. 1h and i). We did not observe the fluorescent signal in cells transfected with the plasmids containing the non-fluorescent fragments of YFP in either cell line (Fig. 1a,b and f,g). Although we show that the interaction of APP with Notch can occur in the absence of presenilin, we cannot rule out the possibility that APP and Notch interactions occur because of other scaffold or adaptor molecules.

### APP inhibits Notch-dependent luciferase activity in HEK293 cells

Following the identification of Notch2 receptor as an APP binding protein, we also demonstrated physical interaction between APP and Notch1 (Oh *et al.* 2005). As Notch2 and Notch1 share a high degree of homology with an amino acid sequence identity of 56% (Weinmaster *et al.* 1992) and because the Notch1 receptors have been the most widely studied, the current research was focused on the functional interaction between APP and the Notch1 receptor. We over-expressed both APP and Notch constructs to determine whether APP can modulate Notch signaling using the Notch-dependent luciferase construct, CBF1-luciferase. The CBF1-luciferase construct (Hicks *et al.* 2000) contains eight tandem repeats of CBF1 DNA binding sites upstream of the luciferase gene, and it has been widely used to study the activation of the Notch signaling pathway. Although we did not activate the over-expressed Notch receptors with the exogenous Notch



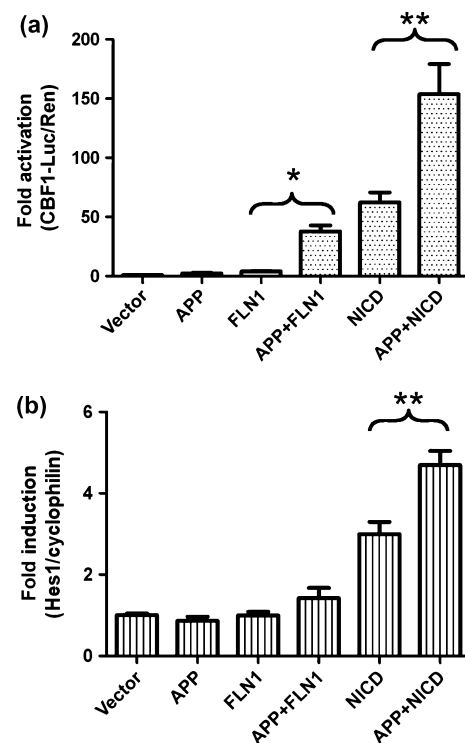


**Fig. 2** APP inhibits Notch signaling in HEK293 cells. HEK293 cells were transiently transfected with plasmids encoding APP, FLN1, NICD together with CBF1-luciferase and phRL-SV40 Renilla plasmids. CBF1-luciferase activity was measured as the ratio of firefly luciferase and Renilla luciferase activity ( $*p < 0.001$ ). The error bars represent standard error of the mean of at least three independent experiments.

ligands, we saw a modest increase in CBF1-luciferase activation in cells transfected with FLN1 compared to empty vector or APP transfected cells (Fig. 2), suggesting that the endogenous Notch ligands present in HEK293 cell are sufficient to activate Notch 1 receptors. This is not surprising as it has been previously shown by western blot of HEK293 cells (Mumm *et al.* 2000), that endogenous Jagged expression was sufficient to activate Notch signaling (shown by S2 and S3 cleavages of exogenously expressed Notch by endogenous Notch ligand, Jagged). We found a marked increase in CBF1-luciferase activation in cells transfected with the constitutively active form of Notch1 receptor, NICD, compared to all other transfection conditions. Interestingly, APP co-transfection showed a significant inhibition of NICD mediated CBF1-luciferase activation (Fig. 2,  $*p < 0.001$ ). Not surprisingly, APP also inhibited Notch2 signaling as demonstrated by inhibition of APP on Notch2-mediated luciferase activity (data not shown). The results from the luciferase assay show that APP negatively modulates Notch1 signaling, which confirms the previous report that APP and Notch compete for the same gamma-secretase activity (Berezovska *et al.* 2001; Lleo *et al.* 2003). However, our data showing that APP also inhibits luciferase activity mediated by the constitutively active form of Notch receptors (NICD) does not fit such a paradigm of competition for the gamma-secretase and suggest that APP interaction with NICD can influence the stability or nuclear translocation of NICD.

#### APP enhances Notch-dependent luciferase activity and further stimulates NICD mediated up-regulation of Hes1 gene expression in COS7 cells

Next, we determined whether the effect of APP on Notch signaling was cell type specific. We found that APP



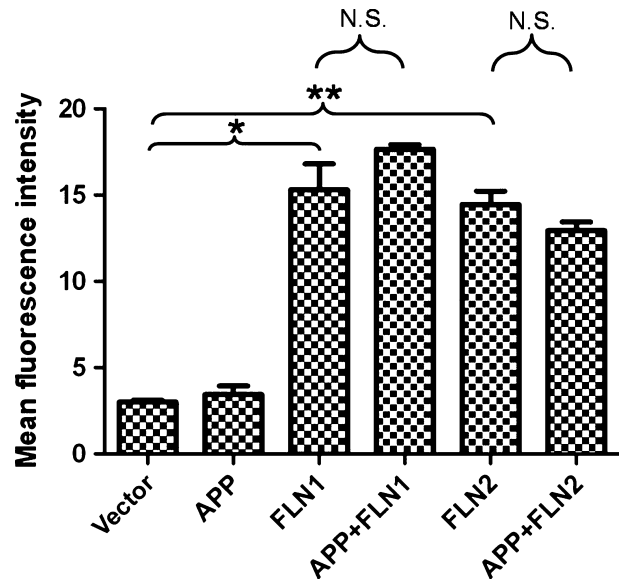
**Fig. 3** APP enhances Notch signaling in COS7 cells. (a) COS7 cells were transiently transfected with plasmids encoding APP, FLN1, NICD along with CBF1-luciferase and phRL-SV40 Renilla plasmid. (b) COS7 cells were transiently transfected with the indicated plasmids. RNA samples were prepared from each transfection, and the endogenous Hes1 mRNA level was measured by qRT-PCR. Data were normalized to cyclophilin mRNA levels. Hes1 mRNA abundance in each transfection was calculated as the relative ratio of each transfection and empty vector control ( $*p < 0.0001$ ,  $**p < 0.005$ ). The error bars represent standard error of the mean (SEM) of at least three independent experiments.

markedly enhanced CBF1-luciferase activity induced by both FLN1 and NICD (Fig. 3a,  $*p < 0.0001$  and  $**p < 0.005$ ). The stimulatory effect of APP on Notch dependent luciferase activity was also seen with Notch2 in COS7 cells (data not shown). We also performed luciferase experiments using APP intracellular domain (AICD), yet, unlike the full-length APP, AICD did not seem to modulate Notch signaling (data not shown). Furthermore, the APP and Notch interaction was not influenced by Notch receptor and ligand interaction as the soluble Notch ligand DIIFc did not have any effect on our luciferase experiments (data not shown). Next, we tested the effect of APP on the endogenous expression of the Notch target gene, Hes1, by quantitative real-time PCR (qPCR) in COS7 cells and found that APP enhanced Hes1 up-regulation mediated by NICD (Fig. 3b,  $**p < 0.005$ ). APP did not show a significant activation of FLN1 mediated Hes1 expression (Fig. 3b) while APP enhanced Notch-dependent luciferase activity (Fig. 3a) Such differences in the effects of APP on FLN receptors between

the luciferase assay and qPCR can be explained by the sensitivity among different assay systems and the degree of the availability of the Notch target gene, *Hes1* (over-expressed in luciferase assay vs. endogenous *Hes1* level in qPCR). We also found that AICD did not play a role in *Hes1* mRNA expression in COS7 cells (data not shown). The ability of APP, but not AICD, to modulate Notch signaling observed in both the luciferase assay and qPCR experiments suggests that both the extracellular and transmembrane domain of APP are necessary to modulate Notch signaling. This result complements our previous work where we showed that both the extracellular and transmembrane domains of Notch are needed for the APP-Notch interaction (Oh *et al.* 2005). As APP inhibited Notch signaling in HEK293 cells shown by the luciferase assay (Fig. 2), we expected that APP would also inhibit *Hes1* mRNA expression. However, the results from these experiments were inconclusive. The same amount of APP and Notch plasmids was used to transfect HEK293 and COS7 for the luciferase assays and qPCR and we found comparable levels of both endogenous and exogenous expression of APP and Notch in the two cell lines (Fig. S1). The results from this western blot analysis rule out the possibility that the enhanced Notch signaling by APP observed in COS7 cells is because of the higher transfection efficiency in COS7 cells.

#### APP does not interfere with the Notch ligand (FcDelta1) binding to Notch receptors in COS7 cells

In our previous study, we hypothesized that the common proteolytic processing pathway shared by Notch and APP may indicate that APP has Notch-like ligands and signaling mechanisms (Oh *et al.* 2005). Thus, we sought to identify APP ligands, which, similar to Delta and Jagged, would induce APP signaling. We identified the Notch receptors as APP-interacting molecules, possibly as novel ligands of APP. As both APP and Notch are transmembrane proteins, bi-directional signaling, as occurring between the ephrin receptor tyrosine kinases and their transmembrane ligands during axon guidance (Egea and Klein 2007), might be the underlying mechanism of interaction between APP and Notch. In order to understand the mechanism by which APP positively regulates Notch signaling in COS7 cells, and test whether APP and Notch function as a receptor/ligand pair, we performed a Notch ligand-binding assay using a soluble form of a Notch ligand, DIIFc, Delta1 fused in frame with the Fc portion of immunoglobulin (Hicks *et al.* 2000). The binding that we observed in cells transfected with the empty vector or APP (instead of Delta and APP) served as the baseline. Although APP did not yield a significant change in DIIFc binding to either Notch1 or Notch2 receptors (Fig. 4), cells transfected with Notch1 or Notch2 alone showed a significant increase in ligand binding (Fig. 4,  $*p < 0.01$  and  $**p < 0.005$ ). Therefore, it is unlikely that APP competes for binding to Notch or

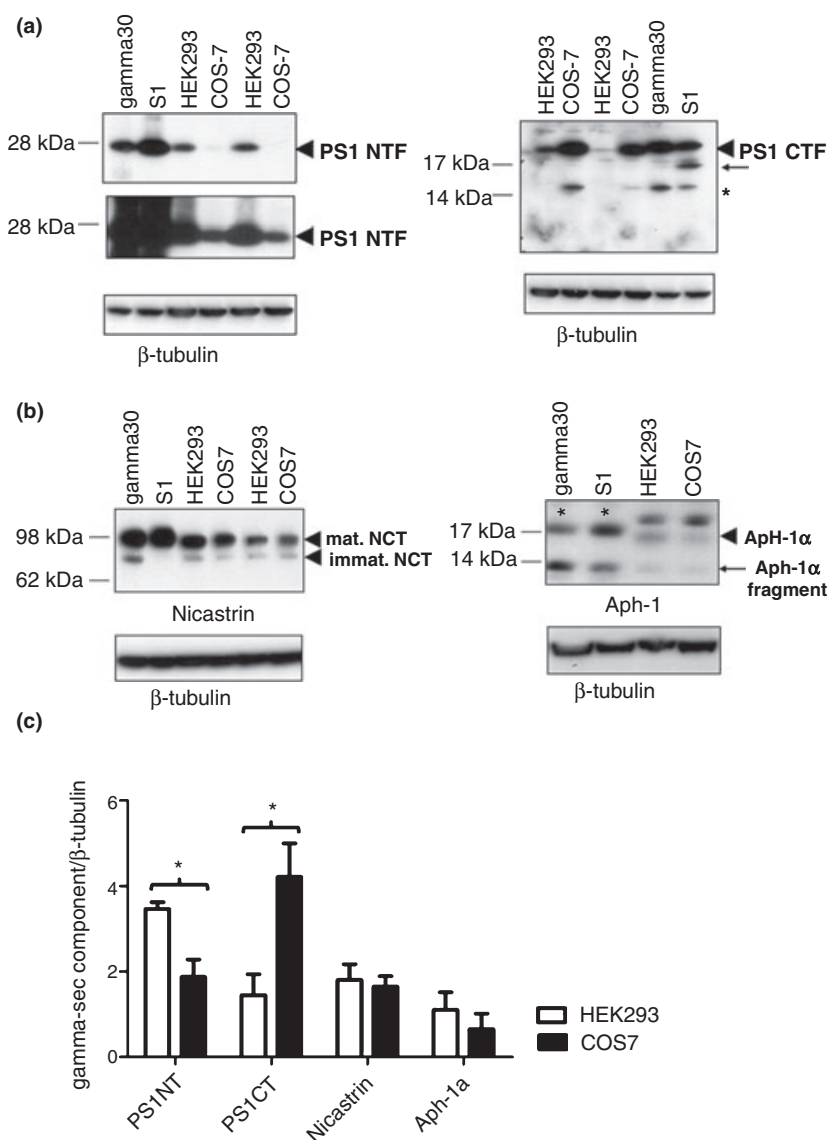


**Fig. 4** APP does not interfere with the Notch ligand FcDelta1 binding to Notch receptors. COS7 cells transiently co-transfected with plasmids encoding APP and FLN1/FLN2 were incubated with CM containing the soluble Notch ligand DIIFc. Binding of DIIFc to Notch proteins was measured by fluorescence-activated cell sorting (FACS) analysis. Data shown are the mean fluorescence intensity  $\pm$  SEM from six independent experiments, each done in triplicate. NS, not significant,  $*p < 0.01$ ,  $**p < 0.005$ .

enhances Notch signaling by modulating the Notch ligand binding in COS7 cells.

#### The endogenous levels of gamma-secretase components are compared in HEK293 vs. COS7 cells by western blot analysis

The cell type specific results that we found with the luciferase assays done in HEK293 vs. COS7 cells may be a consequence of different level of gamma-secretase components in these two cell lines. Therefore, we sought to determine the level of gamma-secretase in each cell line by western blot to explain the differential effects of APP on Notch signaling. Two CHO cell lines stably over-expressing each component of the gamma-secretase complex, gamma30 and S1 cells, served as positive controls. Cell lysates from HEK293 and COS7 cells were analyzed for PS1CTF, PS1NTF (Fig. 5a), Nicastrin, or Aph-1 $\alpha$  (Fig. 5b). We found that the endogenous PS1NTF was more abundant in HEK293 cells compared to COS7 cells (Fig. 5a, left panel). The monoclonal rat antibody for the PS1NTF used in our study, MAB1563 (Chemicon), recognizes  $\sim 28$  kDa NTF of PS1, as expected. The level of endogenous PS1NTF in HEK293 cells is  $\sim 2$ -fold higher than in COS7 cells (Fig. 5c). We then determined the level of PS1CTF. COS7 cells expressed higher level of PS1CTF compared to HEK293 cells, and interestingly, the level of PS1CTF in COS7 cells was



**Fig. 5** Comparisons of gamma-secretase components in HEK293 vs. COS7 cells. The endogenous levels of different components of gamma-secretase were compared in HEK293 vs. COS7 cells. Gamma30 cells and S1 cells were included as positive controls. Detergent-solubilized cell lysates were analyzed by SDS-PAGE and western blot using an antibody recognizing (a) N-terminal (PS1NTF) and C-terminal fragments (PS1CTF) of presenilin 1. *Left*: PS1NTF in COS7 cells is readily detectable only with the longer exposure (bottom panel). *Right*: WB with PS1 C-terminal antibody. Additional fragments of PS1CTF (arrow and asterisk) were detected. (b) Lysates were also analyzed by western blot using antibodies against nicastrin and Aph-1.  $\beta$ -tubulin was used as a loading control. (c) Quantification of gamma-secretase components in HEK293 vs. COS7 cells from the western blot normalized to beta-tubulin levels. \* $p < 0.05$ .

comparable to the two cell lines (gamma30 and S1) that over-express PS1 (Fig. 5a, right panel). A previous study done in COS7 cells showed that the level of PS1CTF was higher in the mock-transfected cells than in cells transfected with PS1 (Tomidokoro *et al.* 1999). We found additional bands in the two CHO cell lines over-expressing PS1 (gamma30 and S1) and in COS7 cells (Fig. 5a, asterisk, right panel). The ~15 kDa band might represent the caspase-cleaved form of PS1CTF (Tesco *et al.* 1998).

We also determined the level of the other two gamma-secretase components, NCT (Fig. 5b, left panel) and Aph-1 (Fig. 5b, right panel). We found a similar level of both the immature and mature nicastrin in HEK293 and COS7 cells (Fig. 5b, left panel). S1 cells stably over-express nicastrin (NCT-GST), and it seems likely that the stable over-expression of NCT favors the mature form of NCT as shown

by the marked reduction in the immature NCT. As NCT has been reported to play a role as a substrate receptor for gamma-secretase (Shah *et al.* 2005), our results showing the similar level of NCT present in HEK293 and COS7 cells suggest that both cell lines exhibit comparable levels of the substrate recognizing capacity of gamma-secretase. Next, we examined, Aph1 $\alpha$  (Fig. 5b, right panel). We observed a ~18 kDa HA-tagged Aph1 $\alpha$  in the two CHO cell lines over-expressing Aph1 $\alpha$ 2-HA (Fig. 5b, right panel, lanes 1 and 2, asterisks). HEK293 cells expressed slightly more Aph1 compared to COS7 cells. We also detected a ~14 kDa Aph1 $\alpha$  fragment in all cell lines, and this Aph1 $\alpha$  fragment has been shown to be present in transfected cells and associated with the gamma-secretase activity (Kimberly *et al.* 2003). Taken together, the differences in the level of each gamma-secretase component in HEK293 vs. COS7

provide supporting evidence for the differential modulation of Notch signaling by APP. The different levels of gamma-secretase components found between HEK293 and COS7 cells might explain the opposite effects of APP in modulating Notch signaling in these two cell lines. However, we also wanted to investigate the activity of the gamma-secretase complex because the higher level of the enzyme expression as seen by western blot does not necessarily correspond to the higher level of activity. Crude plasma membrane fractions containing the active gamma-secretase complex were prepared from HEK293 and COS7 cells. The membrane preparation from gamma30 cells over-expressing components of gamma-secretase was also analyzed as a positive control. The gamma-secretase activity measured in COS7 cells was ~15% higher than HEK293 cells (Fig. S2). The higher level of presenilin C-terminal fragment (Fig. 5b and c), which possesses the catalytic activity of gamma-secretase supports the results from the activity assay suggesting that COS7 cells have higher gamma secretase activity than HEK293 cells. The activity in gamma30 cells was higher than COS7 and HEK293 cells, by 75% and 106%, respectively. The results from the current study demonstrated that the level of gamma-secretase activity is an important factor to determine whether APP functions as a positive or negative modulator of Notch signaling.

#### Both APP and the AICD enhance the nuclear localization of NICD in COS7 cells

We have found that APP enhances Notch signaling in Notch-dependent luciferase assays and qPCR of the Notch target gene, *Hes1* in COS7 cells (Fig. 3). We next sought to determine the mechanism underlying the enhancement of Notch signaling by APP in COS7 cells. The questions that we wanted to address were: (i) Does APP increase the stability of NICD via interaction with already-known cytosolic adaptor proteins, like Numb, or yet unidentified modulators of the Notch signaling pathway? (ii) Does APP increase the nuclear translocation of NICD upon its release from the cell membrane by aiding the nuclear entry? To determine whether the direct interaction of APP or AICD with NICD can account for the predicted alterations in NICD stability and localization, we first performed co-immunoprecipitation (co-IP) experiments to test the physical binding between APP/AICD and NICD. We transfected COS7 cells with APP or AICD together with NICD or FLN1. In our co-IP experiment, we did not detect the binding of APP or AICD to NICD (data not shown). These results are consistent with our previously published study where we could not co-IP NICD with APP (Oh *et al.* 2005). We next tried to determine whether APP or AICD regulates the stability of NICD by co-transfecting cells with APP or AICD and NICD. We did not find a significant difference in the level of NICD by western blot (data not shown), which suggests that neither APP nor AICD affect

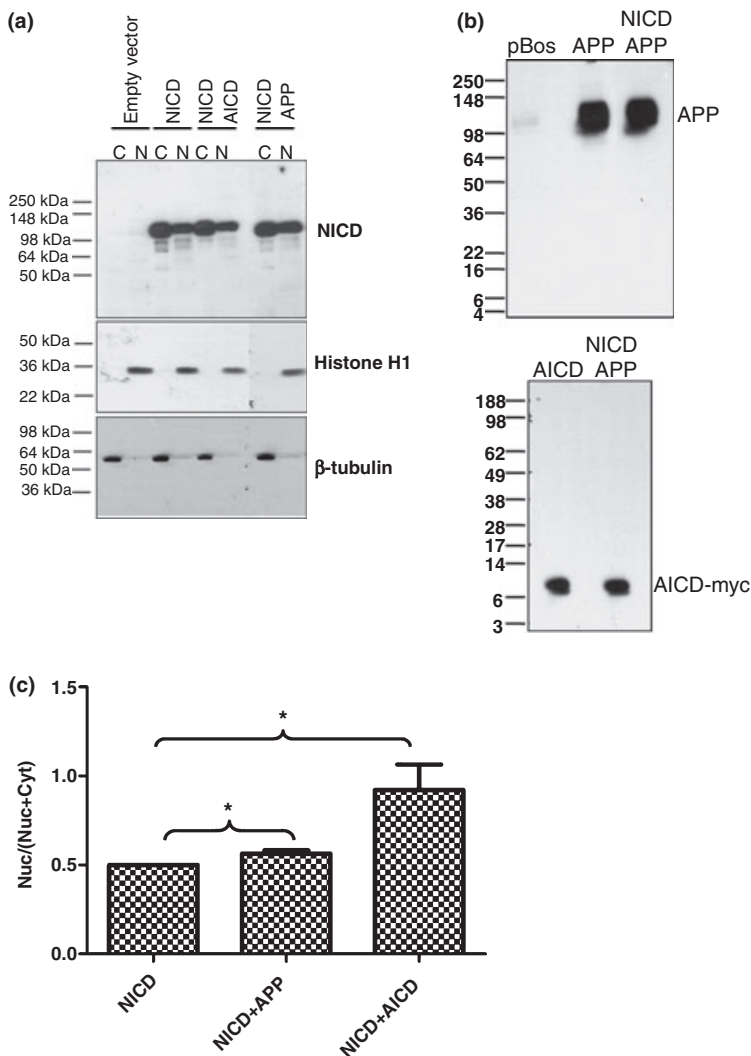
the overall steady-state level of NICD. We also addressed the possibility that APP/AICD can affect NICD trafficking to the nucleus. As shown in Fig. 6(a), we found NICD bands migrating with the molecular weight of ~100–120 kDa using a NICD specific antibody in cells transfected with NICD. The amount of NICD in the nuclear fraction was quantified from the ratio of nuclear NICD and total NICD (sum of nuclear and cytosolic NICD) (Fig. 6c). We found that both APP and AICD increased nuclear NICD, suggesting that APP and AICD can enhance nuclear trafficking or stability of nuclear NICD by a yet unknown mechanism. Although we transfected cells with FLN1 and tried to determine the level of NICD derived from FLN1 by western blot, the intensity of NICD band was too low to be quantified (data not shown).

#### Discussion

The discovery of APP and Notch interaction (Oh *et al.* 2005; Chen *et al.* 2006), led us to examine the functional relevance of this interaction. First, we tested whether the gamma-secretase proteolytic complex, which plays a crucial role in Notch signaling by releasing the active form of the Notch receptors, NICD, and in APP processing, by releasing AICD, acts as a scaffold to link its two substrates, APP and Notch. We demonstrate using BiFC experiments in PSdKO cells that APP and Notch interact in the absence of presenilin. Studies by other groups showing that APP and Notch are processed by gamma-secretase in different subcellular locations (Tarassishin *et al.* 2004), suggested that these two substrates do not compete for the same gamma-secretase activity (Chen *et al.* 2001) provide further evidence supporting our BiFC results. However, other studies reported that APP and Notch compete for gamma-secretase cleavage (Berezovska *et al.* 2001). When APP and Notch interaction occurs as shown by the formation of APP-Notch heterodimers in our BiFC study, a novel proteolytic activity of gamma-secretase unique to the cleavage of such heterodimers may exist. Also, our findings of gamma-secretase-independent interaction between APP and Notch suggest that, the signaling pathways mediated by the APP-Notch heterodimers might be different from the canonical Notch and APP intracellular pathways, and may circumvent the requirement for gamma-secretase. In fact, gamma-secretase-independent pathways have been reported for both APP (Hass and Yankner 2005) and Notch (Berechid *et al.* 2002) using the same PSdKO cells utilized in our study. Therefore, our BiFC study provides novel perspectives to the current paradigm of regulated intramembrane proteolysis governing APP and Notch processing by gamma-secretase, and suggests that APP-Notch heterodimers might be processed by, as yet unidentified, proteolytic enzymes.

As the Notch signaling pathway has been extensively studied using the Notch-responsive reporter systems com-





**Fig. 6** APP/AICD enhances NICD nuclear localization in COS7 cells. (a) COS7 cells were transiently transfected with the plasmids indicated. Cells were harvested 48 h post-transfection, and nuclear and cytosolic fractions were prepared from the cell lysates. The level of Notch intracellular domain (NICD) was analyzed by western blot using a NICD-specific antibody. The histone H1 immunostaining served as a nuclear marker, whereas  $\beta$ -tubulin staining was used as a cytosolic marker. (b) COS7 cells were transfected with NICD alone or co-transfected with APP or AICD-myc plasmids. Cell lysates were harvested and analyzed by western blot for APP (anti-APP antibody, 6E10, left panel) or AICD-myc (anti-cMyc antibody, right panel). (c) The nuclear localization of NICD was quantified as a ratio between the nuclear NICD and total NICD (nuclear + cytosolic). Each fraction was normalized by nuclear (histone H1) or cytosolic ( $\beta$ -tubulin) markers; N, nuclear fraction; C, cytosolic fraction. Error bars represent standard error of the mean from three independent experiments; \* $p < 0.05$ .

pared to the somewhat artificial APP-dependent reporter systems (Cao and Sudhof 2001; Andersen *et al.* 2005), we performed Notch-dependent luciferase assays. Our data from the luciferase assays and qPCR, showed that APP differentially modulates Notch signaling depending on the cell types. The fundamental differences between the two cell lines used in our study lie in their origin; HEK293 cells are derived from the kidney cells of a human fetus (Graham *et al.* 1977) whereas COS7 cells are derived from kidney cells of the African green monkey (Jensen *et al.* 1964). Therefore, the difference in various aspects of cellular environments especially with respect to Notch signaling could be considered. Also, as both APP and Notch have been shown to interact with various other signaling pathways, it is not surprising that we observed that APP differentially modulates Notch signaling. The levels of proteolytic enzymes, which govern APP and/or Notch processing, cross-talks with other signaling pathways, Notch ligands, adaptor proteins, and other known modulators of Notch signaling pathways could

vary in different cell types. In fact, modulation of Notch signaling pathway by APP can be regulated at multiple levels. For example, regulation of Notch ligands by other signaling pathways could provide potential insights on the cell-type specific, differential modulation of APP on Notch signaling. Although the cell-type specificity has not been studied, glycosylation of Notch receptors by fringe differentially modulates Notch signaling by altering the strength of Notch ligand-receptors interactions (Hicks *et al.* 2000; Yang *et al.* 2005). Also, the effects of APP on Notch signaling taken from our data, at least in COS7 cells, may involve a cytosolic adaptor protein, Numb, an endocytic adapter protein and Notch inhibitor (Roncarati *et al.* 2002). As the AICD binds Numb, when APP is over-expressed in our luciferase assays, APP/AICD might sequester Numb molecules away from Notch, thus repressing the Notch inhibition. Also, a recent study reported that the alternatively spliced Numb isoforms affect intracellular APP trafficking (Kyriazis *et al.* 2008). Thus, in our study, the different cellular

environments in HEK293 vs. COS7 cells might account for potential variations in APP and Numb interactions; thereby resulting in differential modulation of Notch signaling. A previous study reported a cell-type-specific processing of APP by PS during *Drosophila* development (Loewer *et al.* 2004). APP processing by PS-dependent gamma-secretase activity was up-regulated in neurons compared to other tissues. Nevertheless, the authors were unable to provide a mechanistic understanding of their observation. Another possibility of cell-type-dependent modulation of APP on Notch signaling could be mediated through alterations in substrate specificity by the gamma-secretase complex. The study of gamma-secretase modulators is an active area of research for therapeutics for AD, cancer, and other diseases. Such a modulator, Rac1, when activated, preferentially mediates gamma-secretase processing of APP compared to Notch (Boo *et al.* 2008).

The cell-type dependent interaction of APP with Notch suggests that the different cellular contexts during development and aging *per se* can elicit divergent consequences of APP and Notch interactions. Emerging evidence of APP and Notch governing neurogenesis has been documented in a number of studies. APP has been shown to modulate neurogenesis via interactions with SORLA (sortilin-related receptor with A-type repeats). SORLA plays a role in the intracellular trafficking of APP among different compartments, including Golgi, plasma membrane, and endosomes (Andersen *et al.* 2005; Offe *et al.* 2006). Yet, another molecule, transient axonal glycoprotein 1, a member of F3/contactin family, has been shown to interact with APP and inhibit embryonic neurogenesis at embryonic day 14 (E14) mouse brain (Ma *et al.* 2008). Notch signaling is implicated in neurogenesis where it plays a role in maintaining the multipotent neural progenitor cells during embryonic neural development (Hitoshi *et al.* 2002). Conversely, aberrant activation of Notch signaling inhibits neurogenesis in a mouse pluripotent embryonic carcinoma cell line, P19 (Jin *et al.* 2004). Therefore, a compelling body of evidence supports the important regulatory functions of both APP and Notch in neurogenesis. In this regard, our results demonstrating that APP differentially modulates Notch can have important implications in the context of neurogenesis in aging and in AD. APP can enhance Notch signaling to maintain the neural progenitor pools by inhibiting neuronal differentiation to keep a sufficient reservoir for the prospective neurogenesis when the need for neurogenesis is called upon, as found in stroke (Zhang *et al.* 2008) or AD (Jin *et al.* 2004; Nagy 2005). The negative modulation of APP on Notch signaling can be particularly beneficial in pathological conditions such as brain tumors, where Notch stimulates abnormal neurogenesis and uncontrolled expansion of stem cells similarly to cancer cell proliferation (Zhang *et al.* 2008). Nevertheless, studies of APP and Notch interaction in the aforementioned clinical settings are required to under-

stand the molecular mechanisms underlying the modulation of neurogenesis.

The demonstration that APP did not alter the Notch ligand binding suggests that APP and Notch interaction exhibits distinct spatiotemporal characteristics compared to the cognate receptor-ligand interactions as occurring in the canonical Notch signaling. For example, APP might modulate downstream signaling events following Notch ligand binding, likely involving interactions with other modulators of Notch signaling. Another plausible explanation is that APP modulates Notch signaling by interacting with Notch within the secretory pathway from the Golgi to the cell surface, or during its internalization via endocytic trafficking by interfering with various endocytic regulators of Notch, including Deltex (Hori *et al.* 2004), Itch/AIP4/Nedd4 (Wilkin *et al.* 2004), Numb (Santolini *et al.* 2000),  $\alpha$ -adaptin (Santolini *et al.* 2000), CCT-1 (Weber *et al.* 2003), Tsg101 (Moberg *et al.* 2005), and Vps25 (Thompson *et al.* 2005).

We showed by western blot that the levels of gamma-secretase components are different between HEK293 and COS7 cells. In particular, the discrepancy in the levels of PS1NTF and PS1CTF is noteworthy. One possibility is that the stability of PS1NTF and PS1CTF is regulated by specific factors in each cell line. For example, the glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) has been shown to phosphorylate PS1CTF and decrease its turnover without affecting the level of PS1NTF (Kirschenbaum *et al.* 2001). Thus, it is possible that COS7 cells have higher levels of GSK-3 $\beta$  or hyperactivation of GSK-3 $\beta$ . Although we cannot explain the higher level of PS1NTF present in HEK293 compared to COS7 cells and the discrepancy between the levels of PS1CTF and NTF in the two cell lines, recent reports (Bergman *et al.* 2004; Shiraishi *et al.* 2006) provide evidence that PS1CTF is required and sufficient for gamma-secretase activity. Also, the exogenous expression of PS1CTF in PS-null cells allowed a reconstitution of gamma-secretase activity (Shiraishi *et al.* 2006), supporting the importance of PS1CTF in the catalytic activity of the gamma-secretase complex. Consistent with the higher PS1CTF level in COS7 cells, we observed a higher level of gamma-secretase activity in COS7 cells compared with HEK293 cells (Fig. S2). Although we showed that APP and Notch interaction does not require gamma-secretase by BiFC (Fig. 1), the data from the expression level and activity of gamma-secretase (Fig. 5) suggest that APP and Notch interaction can be regulated by gamma-secretase-independent and/or dependent mechanisms.

Finally, our results showing that APP and AICD enhance the nuclear localization of NICD in COS7 cells without affecting the steady state level of NICD suggest that APP and/or AICD might promote nuclear translocation of NICD. Although we considered a possibility that the binding of APP/AICD to NICD facilitates the nuclear localization of

NICD, we did not find evidence of the binding interaction between APP/AICD and NICD, which is in contrast with a recent study done in HEK293 cells showing a direct binding of AICD and NICD, both in the cytosol and nucleus by co-IP experiments (Kim *et al.* 2007). Therefore, a determination of the molecular mechanism of the enhanced nuclear localization of NICD by APP/AICD would be of importance to understand the underlying mechanisms of cell-type dependent modulation of APP on Notch signaling. We found that AICD failed to modulate Notch signaling by luciferase assays and qPCR of Hes1 mRNA expression (data not shown), yet AICD was shown to enhance NICD translocation to the nucleus in COS7 cells. This suggests that although AICD may not play a role in modulating NICD mediated transcriptional regulation it may regulate other processes relevant to the NICD stability, such as ubiquitination of NICD (Oberg *et al.* 2001). Our study presents a novel finding that APP and Notch interaction can occur in the absence of the gamma-secretase complex. Yet, APP-Notch interaction can be differentially regulated by the levels of gamma-secretase activity in a cell-type specific manner. It will be interesting to investigate the changes in the level of gamma-secretase activity in the brain during development, aging, and disease pathogenesis, and the mode of APP and Notch interaction in each process. In conclusion, APP and Notch interaction can occur in the presence and/or absence of active gamma-secretase complex, so the future studies aiming at identifying the underlying mechanism of APP and Notch interactions in gamma-secretase-independent vs. gamma-secretase-dependent pathways may have a great therapeutic potential in the pathogenesis of various diseases including AD and cancer.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Materials and methods.

**Figure S1.** Co-transfection of APP and Notch plasmids in HEK293 and COS7 cells result in similar levels of expression of APP, Notch1, and Notch2 proteins.

**Figure S2.** Comparison of *in vitro* gamma-secretase assay in HEK293, COS7, and gamma30 cells.

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