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DAB1 and Reelin Effects on Amyloid Precursor Protein and ApoE Receptor 2 Trafficking and Processing*

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Numerous cytoplasmic adaptor proteins, including JIP1, FE65, and X11α, affect amyloid precursor protein (APP) processing and Aβ production. Dab1 is another adaptor protein that interacts with APP as well as with members of the apoE receptor family. We examined the effect of Dab1 on APP and apoEr2 processing in transfected cells and primary neurons. Dab1 interacted with APP and apoEr2 and increased levels of their secreted extracellular domains and their cytoplasmic C-terminal fragments. These effects depended on the NPXY domains of APP and apoEr2 and on the phosphotyrosine binding domain of Dab1 but did not depend on phosphorylation of Dab1. Dab1 decreased the levels of APP β-C-terminal fragment and secreted Aβ. Full-length Dab1 or its phosphotyrosine binding domain alone increased surface levels of APP, as determined by surface protein biotinylation and live cell staining. A ligand for apoEr2, the extracellular matrix protein Reelin, significantly increased the interaction of apoEr2 with Dab1. Surprisingly, we also found that Reelin treatment significantly increased the interaction of APP and Dab1. Moreover, Reelin treatment increased cleavage of APP and apoEr2 and decreased production of the β-C-terminal fragment of APP and Aβ. Together, these data suggest that Dab1 alters trafficking and processing of APP and apoEr2, and this effect is influenced by extracellular ligands.

Proteolysis of the amyloid precursor protein (APP)[2] results in production of the Aβ peptide, found in plaques of Alzheimer disease. The cytoplasmic domain of APP contains an NPTY sequence that serves as a binding motif for adaptor proteins that possess a phosphotyrosine binding domain (PTB), such as members of the Fe65, X11, JIP, and Dab protein families. Such adaptor proteins play critical roles in tyrosine kinase-mediated signal transduction, protein trafficking and localization, phagocytosis, cell fate determination, and neuronal development (1). Most importantly for Alzheimer disease, interactions between these cytoplasmic proteins and APP also lead to altered processing of APP.

The effects of several of these adaptor proteins on APP trafficking and processing have been studied. The Fe65 family (FE65, FE65L1 (2), and FE65L2 (3)) is expressed at high levels in neurons (4). Co-expression of FE65 and APP promotes secreted APP and Aβ secretion in H4 cells and Madin-Darby canine kidney cells (2). However, co-expression of FE65 and APP in HEK293 cells stabilizes immature APP and inhibits APPs formation and Aβ secretion (5). The X11 family (X11α, -β, and -γ; also called Mint1, -2, and -3) are important in neuronal synaptic function (6). X11α slows cellular APP processing and reduces Aβ40 and Aβ42 secretion, perhaps through preventing trafficking of APP to subcellular compartments containing active γ-secretase (7). X11β reduces Aβ levels and amyloid plaque formation in the brains of transgenic APP mice (8). Similar to X11α and -β, JIP-1b interaction with APP stabilizes immature APP and inhibits APPs, Aβ40, and Aβ42 secretion *in vitro* (9).

In contrast, the effects of the Dab family (Dab1 and Dab2) on APP processing are less well studied. Dab1 is important for nervous system development in mammals (10). Dab1 and Dab2 possess a PTB domain and have been shown to interact with the cytoplasmic domains of APP and apoE receptors (apoEr2, VLDLr, and low density lipoprotein receptor-related protein) (11, 12). The extracellular domains of apoEr2 and VLDLr interact with extracellular matrix protein Reelin during neuronal positioning (13). Reelin induces Dab1 tyrosine phosphorylation (13). If any of these components (Dab1, apoE receptors, or Reelin) is knocked out, a similar phenotype of impaired neuronal migration is observed.

In this study, we demonstrate that Dab1 increases cell surface expression of APP and apoEr2, increases α-cleavage of APP and apoEr2, and decreases APP β-CTF and Aβ in transfected cells and in primary neurons. We found that Reelin treatment significantly increases co-immunoprecipitation of Dab1 with both APP and apoEr2. Moreover, Reelin, like Dab1, increases cleavage of APP and apoEr2 and decreases production of the β-CTF of APP and Aβ.

MATERIALS AND METHODS

Vector Construction—The following Dab1 constructs were produced by PCR and inserted into the PCMV-Tag4 expression vector (Stratagene) to FLAG tag the C termini (Fig. 1): Dab1-1

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[2] The abbreviations used are: APP, amyloid precursor protein; apoE, apolipoprotein E; apoEr2, apoE receptor 2; PTB, phosphotyrosine binding; VLDLr, very low density lipoprotein receptor; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HBSS, Hanks' balanced saline solution; AICD, APP intracellular domain; sapo, secreted apo; sAPP, secreted APP; CTF, C-terminal fragment; ECS, extracellular solution.

(residues 1–166), Dab1-2 (residues 167–555), and Dab1-3 (full-length, residues 1–555). ApoEr2 with a C-terminal HA tag was generated as described (14). We produced an NPVY mutant of apoEr2 using site-directed mutagenesis, substituting alanine for asparagine. Wild-type APP770 and an APP NPTY mutant (APTY) with C-terminal Myc tags were the kind gifts of Dr. Brad Hyman. Recombinant DNA was confirmed by sequencing, and expression of correctly sized proteins was confirmed by Western blot.

Cell Lines and Culture Conditions—COS7 and HEK293 were maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen) in a 5% CO_2 incubator. COS7 cells were transiently transfected with 0.5–1 μg of plasmid in FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol and cultured 24 h in Dulbecco's modified Eagle's medium containing 10% FBS. For co-transfections, cells were similarly transfected with 0.5–1 μg of each plasmid in FuGENE 6 (Roche Applied Science) and cultured for 24 h in Dulbecco's modified Eagle's medium with 10% FBS. After 24 h the cells were transferred to Opti-MEM serum-free media (Invitrogen) and treated with indicated compounds. The Reelin-conditioned and control media were prepared and concentrated 10 times as described (15). Conditioned media were incubated at a 1:10 dilution in serum-free media on primary neuronal cells for 30 min.

Antibodies—We used antibodies anti-HA (Abcam), anti-FLAG (Sigma), anti-c-Myc (Abcam), and anti-Dab1 (from Dr. Andre Goffinet). Antibody 5810 (against the N terminus of apoEr2) was the kind gift of Dr. Uwe Beffert. For analysis of APP, we used 6E10 (identifying sAPPα) (Signet), 22C11 (Chemicon), and c1/6.1 (recognizing the C terminus of APP), provided by Dr. Anne Cataldo.

Quantification of APP and ApoEr2 Proteolytic Fragments—Secreted fragments were identified by Western blot analysis of conditioned media (sAPPα, 6E10 antibody; sapoEr2, 5810 antibody). CTF were measured by Western blots of cell lysates (APP α-CTF, C1/6.1; APP βCTF, 6E10; apoEr2 CTF, HA antibodies). Aβ40 and Aβ42 levels in the conditioned media were determined by ELISA, using 1A10 (anti-Aβ40) or 1C3 (anti-Aβ42) as capture antibodies and 12B2, which recognizes both mouse and human Aβ as the detection antibody (Immuno-Biological Laboratories) (16).

Primary Neuronal Cell Culture—Primary mouse embryonic cortical neurons were prepared from embryonic day 16 (E16) Swiss-Webster mice as described (17). Brain cortices were chopped and trypsinized for 10 min at 37 °C. After trypsinization, 0.4 μg/ml trypsin inhibitor, 0.025% DNase, and 12 mM $MgSO_4$ were added and mixed until tissue was thoroughly homogenized. Cells were then transferred to Neurobasal medium containing B27 serum supplement, 1 mM glutamine, gentamycin, and Ara-C. Neurons (2×10^6 cells) were seeded on 50 μg/ml poly-D-lysine-coated 12-well tissue culture plates. For analysis of the APP-Dab1 interaction, cells were seeded on 35-mm tissue culture plates. Cells at 10 days *in vitro* were treated with either control or Reelin-containing medium for 30 min. For Dab1 knock-out primary neurons, brain cortices from mouse E16 embryos of $Dab1^{+/-}$ crosses were dissociated separately, and neurons were seeded from each embryo into the pre-coated 24-well tissue cultures. For experiments on the effects of Dab1, neurons derived from individual embryos were used as follows: $Dab1^{+/+}$ ($n = 12$), $Dab1^{+/-}$ ($n = 9$), and $Dab1^{-/-}$ ($n = 8$).

Reelin Treatment of APPsw-TG Primary Neurons—APPsw-TG mice (Tg2576) were obtained from Taconic. APPsw-TG (E16.5) cortices were dissected in Hanks' balanced saline solution (HBSS) and were dissociated with 0.25% trypsin at 37 °C for 15 min. Cells were washed twice with HBSS and dissociated further with DNase with trypsin inhibitors (Roche Applied Science) with a glass pipette. The dissociated cells were washed again in HBSS twice and resuspended in Neurobasal defined media (Invitrogen/Invitrogen) with 1% penicillin/streptomycin/glutamine (Invitrogen) and 4% B-27 supplement. Primary neurons were plated on poly-L-lysine-coated 12-well dishes and grown at 37 °C for 4–5 days. The media were changed for all neurons 1 h before stimulation, and then primary neurons were treated with either control or Reelin-conditioned media (10×) for 18 h. The supernatants were collected, and the levels of secreted Aβ40 were measured by ELISA (BIOSOURCE). Wild-type ($n = 3$) and APPsw-TG ($n = 3$) cortices were used, and three cultures from each animal were treated with Reelin or control media.

Co-immunoprecipitations—Transfected COS7 cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1% Nonidet P-40, phosphatase inhibitors (Sigma), and protease inhibitors (Roche Applied Science). For immunoprecipitation, the lysates were incubated for 2 h at 4 °C with the anti-HA antibody or 6E10 antibody bound to protein G-Sepharose beads (Amersham Biosciences). The precipitates were then washed three times with lysis buffer and resuspended in SDS sample buffer. The samples were separated by SDS-PAGE on 4–15% polyacrylamide gels, transferred electrophoretically to nitrocellulose membranes, and blocked with 5% nonfat dry milk. The blots were incubated with antibodies at room temperature for 1 h. Horseradish peroxidase-conjugated secondary antibodies were visualized by ECL detection system and exposed to film.

Biotin Labeling of Cell Surface Proteins—COS7 cells were transiently transfected with APP and Dab1 constructs or apoEr2 and Dab1 constructs in FuGENE 6 (Roche Applied Science) and cultured for 24 h in Dulbecco's modified Eagle's medium containing 10% FBS. After 24 h, cells were washed twice with PBS, and surface proteins were labeled with Sulfo-NHS-SS-Biotin (Pierce) in PBS under gentle shaking at 4 °C for 30 min. 50 μl of quenching solution was added to cells at 4 °C, which were washed twice with TBS. Cells were collected in 500 μl of lysis buffer, disrupted by sonication on ice, incubated for 30 min on ice, and clarified by centrifugation (10,000 × *g*, 2 min). To isolate biotin-labeled proteins, lysate was added to immobilized NeutrAvidin™ gel (50 μl) and incubated 1 h at room temperature. Gels were washed five times with wash buffer and incubated 1 h with SDS-PAGE sample buffer, including 50 mM dithiothreitol. Eluants were analyzed by immunoblotting.

Live Cell Surface Staining—Transfected HEK293 cells were washed with PBS and incubated with primary antibody diluted in ECS (containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM

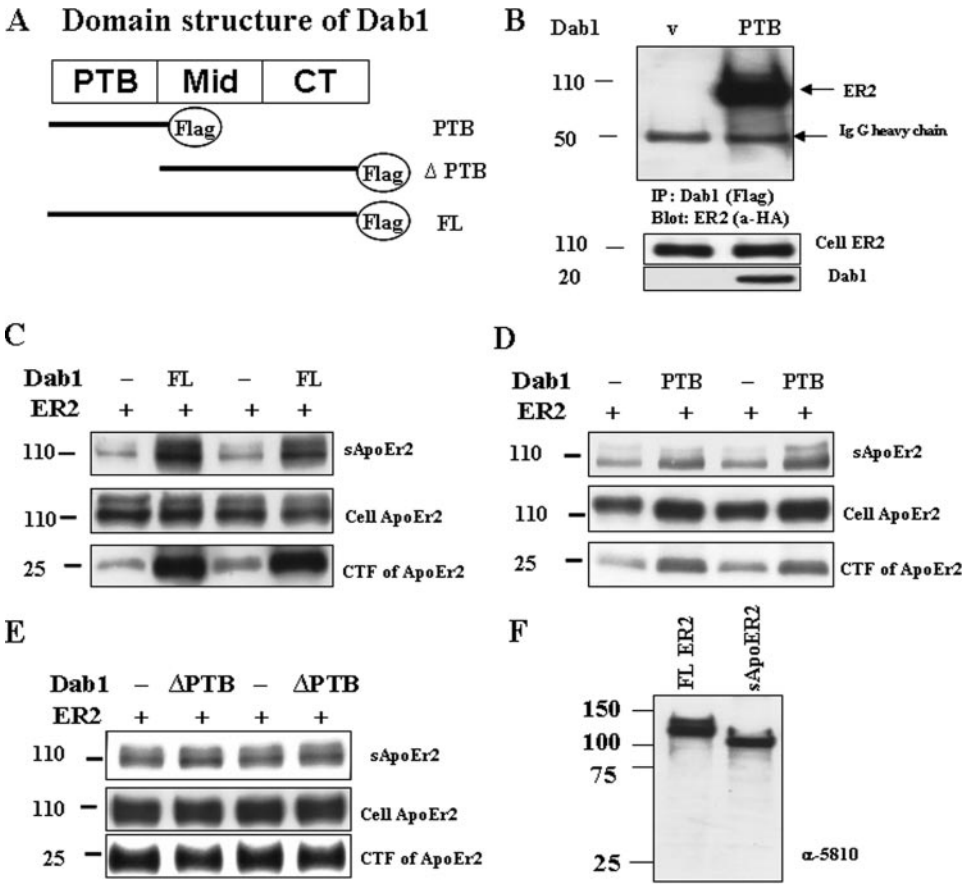

FIGURE 1. **Dab1 alters apoEr2 processing.** *A,* several deletion mutants of C-terminal FLAG-tagged Dab1 were produced as follows: residues 1–166, PTB domain of Dab1; residues 167–555, PTB deletion construct of Dab1 (Δ*PTB*); 1–555, full-length Dab1(*FL*). *B,* COS7 cells were transiently transfected with apoEr2 (HA-tagged) and Dab1 PTB domain. Cell lysates (200 μg) were immunoprecipitated (*IP*) with anti-FLAG, and the precipitate was probed with anti-HA. ApoEr2 co-precipitated with constructs containing PTB domain of Dab1. Lysates from co-transfected cells (20 μg) were probed for apoEr2 or Dab1 to demonstrate expression levels (*lower panel*). *Lane v*, vector alone. *C,* COS7 cells were transiently transfected with apoEr2 with constructs of full-length Dab1. Secreted apoEr2 was measured in conditioned media with antibody 5810, and apoEr2 CTFs were detected from cell lysates with anti-HA antibody. Dab1 increased secreted apoEr2 and apoEr2 CTF. *D,* COS7 cells were transiently transfected with apoEr2 (HA-tagged) and Dab1 PTB. The PTB domain of Dab1 increased secreted apoEr2 and apoEr2 CTF. *E,* COS7 cells were transiently transfected with apoEr2 and Dab1 ΔPTB. The PTB deletion construct of Dab1 did not alter secreted apoEr2 or apoEr2 CTF. *F,* COS7 cells were transiently transfected with apoEr2. Secreted apoEr2 from conditioned media and cell associated apoEr2 from lysates were measured with antibody 5810. The band for cell-associated apoEr2 is slightly higher than that for sapoEr2.

$CaCl_2$, 5 mM HEPES, 5 mM glucose, 15 mM sucrose, 0.25 mg/l phenol red, and 10 μM D-serine (all from Sigma), adjusted to pH 7.4 with NaOH) for 8 min at room temperature. Cells were washed three times in ECS and then incubated with 22C11 antibody for 8 min, washed with ECS, and incubated with Alexa Fluor 488 (Molecular Probes) goat anti-mouse antibody for 8 min at room temperature. Final washes were carried out with ECS. After fixing the cells with 4% paraformaldehyde, cells were imaged on an Axioskop FS microscope (Zeiss, Germany) equipped with a ×63, 0.9 NA, Achroplan, water-immersion objective or with a Nikon E600 microscope (Nikon, Japan) equipped with a ×60, 1.0 NA.

FE65-dependent APP Luciferase Transactivation Assay—The FE65-dependent APP luciferase transactivation assay was performed by the method of Cao and Sudhof (18). HEK293 cells were cotransfected with the APP-Gal4 construct, FE65-myc, and Dab1 constructs, as well as the pG5E1B-luciferase reporter plasmid (to measure activation) and a β-galactosidase plasmid (to normalize transfection efficiency). Luciferase activity from cell lysates was determined in triplicate using the luciferase assay kit (Promega) by VICTOR2 (PerkinElmer Life Sciences). Results were normalized to β-galactosidase expression levels by using a β-galactosidase enzyme assay kit (Promega).

Statistical Analyses—Experiments were repeated a minimum of four times unless otherwise noted. All data were analyzed using analysis of variance with Graphpad Prism 4 software, using Tukey's multiple comparison test for post hoc analyses with significance determined as $p < 0.05$. Student's *t* test was performed on Aβ measures from Reelin-treated primary neurons. Descriptive statistics were calculated with StatView 4.1 and displayed as an expressed mean ± S.E.

RESULTS

Dab1 Interacts with ApoEr2 in COS7 Cells and Affects Its Processing—In order to examine the effects of Dab1 on processing of apoEr2, we generated FLAG-tagged constructs of full-length Dab1, Dab1 lacking the PTB domain, and the PTB domain lacking the C terminus of Dab1 (Fig. 1*A*). Proteins from each construct were successfully expressed in COS7 cells with full-length apoEr2 (data not shown). We tested for interactions between the PTB domain of Dab1 and apoEr2 by immunoprecipitating the Dab1 PTB domain with an anti-FLAG antibody and probing for apoEr2 with the anti-HA antibody. ApoEr2 co-precipitated with the PTB domain of Dab1 but did not precipitate in the absence of Dab1 (Fig. 1*B*, *upper panel*). Levels of total apoEr2 and Dab1 were consistent across transfections (Fig. 1*B*, *middle* and *lower panels*). We also immunoprecipitated apoEr2 with the anti-HA antibody and found that the Dab1 PTB construct co-precipitated (data not shown).

We tested whether the interaction of Dab1 with apoEr2 affected processing of apoEr2. ApoEr2 is cleaved similarly to APP, with an α-secretase-like activity generating a secreted form of the receptor and a membrane-bound CTF (14). In addition, γ-secretase activity cleaves the apoEr2 CTF (14). COS7 cells were transfected with full-length apoEr2 alone or apoEr2 with Dab1 constructs lacking the C terminus of Dab1 or lacking the PTB domain. Secreted apoEr2 was measured in conditioned media, and apoEr2 CTF was measured in cell lysates. Full-length Dab1 significantly increased secreted apoEr2 and apoEr2 CTF (Fig. 1*C*). Dab1 expression did not alter cell apoEr2

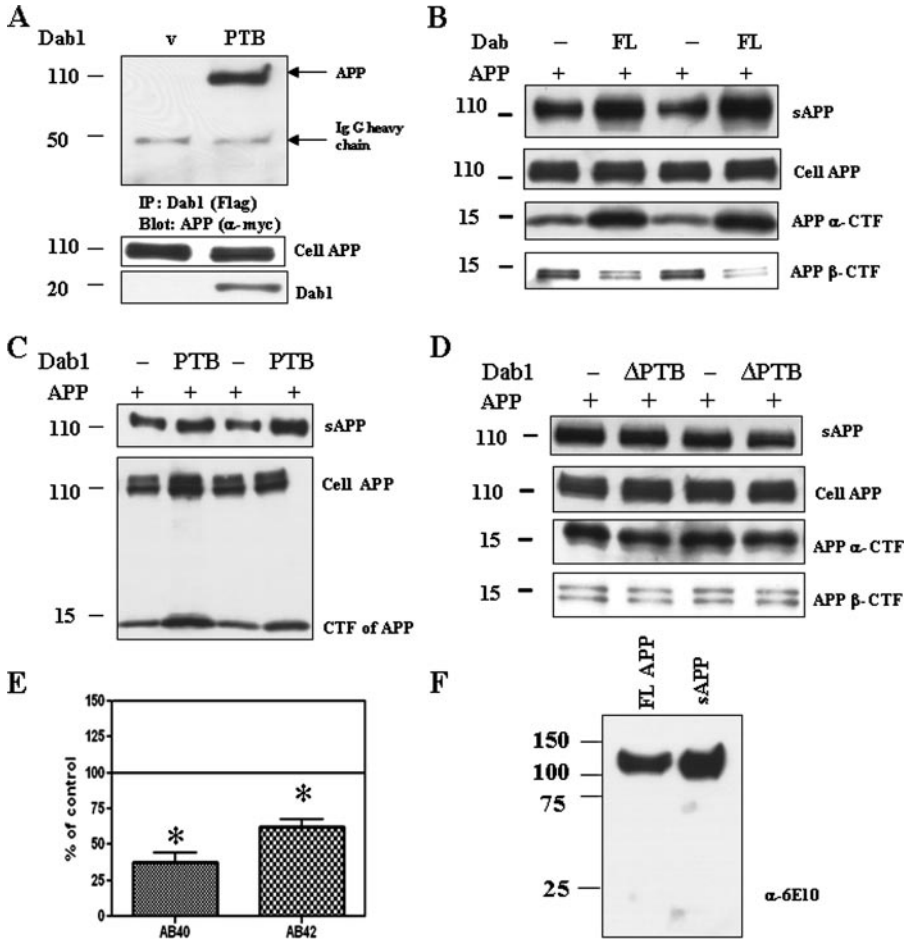

FIGURE 2. **Dab1 alters APP processing.** *A*, COS7 cells were transiently transfected with APP (Myc-tagged) and Dab1 PTB. Cell lysates (200 μg) were immunoprecipitated with anti-FLAG, and the precipitate was probed with anti-Myc. APP co-precipitated with the Dab1 PTB domain in COS7 cells. Lysates from co-transfected cells (20 μg) were probed for APP or Dab1 to demonstrate expression levels (*lower panel*). *Lane v*, vector alone. *B*, COS7 cells were transiently transfected with APP with constructs of full-length Dab1. Conditioned media were analyzed for secreted forms of APP (6E10); cell lysates were analyzed for α-CTF of APP (c1/6.1) and β-CTF of APP (6E10). Full-length Dab1 increased secreted forms and α-CTF of APP but decreased APP β-CTF. *C*, COS7 cells were transiently transfected with APP and Dab1 PTB. The PTB domain of Dab1 increased secreted forms and α-CTF of APP. *D*, COS7 cells were transiently transfected with APP and Dab1 ΔPTB. Conditioned media were analyzed for secreted APP (6E10); cell lysates were analyzed for α-CTF of APP (c1/6.1) and APP β-CTF (6E10). The Dab1 PTB deletion constructs did not alter secreted APP or APP. *E*, COS7 cells were transiently transfected with APP and vector or APP and full-length Dab1. Aβ40 and Aβ42 levels in the conditioned media were determined by ELISA. Full-length Dab1 decreased secreted Aβ40 levels (by 68%, $p < 0.05$) and Aβ42 levels (by 44%, $p < 0.05$). *F*, COS7 cells were transiently transfected with APP. Secreted APP and cell associated APP was measured with antibody 6E10. The band for cell-associated APP is slightly higher than that for sAPP.

levels (Fig. 1*C*). We also found that the PTB domain of Dab1 increased secreted apoEr2 and apoEr2 CTF (Fig. 1*D*). The PTB domain of Dab1 expression also slightly increased cell apoEr2 levels (Fig. 1*D*). Because Dab1 interacts with apoEr2 through its PTB domain (19), we hypothesized that constructs of Dab1 lacking the PTB domain would not affect apoEr2 processing. We found that PTB deletion construct of Dab1 did not affect sapoEr2 or apoEr2 CTF levels (Fig. 1*E*). Thus, Dab1 interactions with apoEr2 promote the cleavage of apoEr2, and this effect depends on the Dab1 PTB domain.

To test the nature of soluble and cell-associated protein bands, we transfected apoEr2 and measured the cell-associated apoEr2 and secreted apoEr2. We found that the band for cell-associated apoEr2 migrated slightly higher than that for sapoEr2 (Fig. 1*F*).

Dab1 Interacts with APP in COS7 Cells and Affects Its Processing—Dab1 was reported to interact with APP by glutathione *S*-transferase pulldown, yeast two-hybrid, co-immunoprecipitation, and x-ray crystallography (11). We tested whether Dab1 had a similar effect on APP processing as it had for apoEr2. We transfected COS7 cells with APP and the Dab1 PTB domain, immunoprecipitated Dab1 PTB with the anti-FLAG antibody, and probed for APP with the anti-Myc antibody. APP co-precipitated with the PTB domain of Dab1 but did not precipitate in the absence of Dab1 (Fig. 2*A*, *upper panel*). We made similar observations with full-length Dab1 (data not shown). Western blot analysis of COS7 cell extracts confirmed similar levels of cell APP and Dab1 across transfections (Fig. 2*A*, *middle* and *lower panels*).

We tested whether the interaction of Dab1 with APP affected processing of APP in COS7 cells. Expression of full-length Dab1 significantly increased secreted APP and APP α-CTF levels but significantly decreased APP β-CTF levels (Fig. 2*B*). Thus, Dab1 co-expression promoted the α-cleavage of APP and decreased its β-cleavage. We also found that the PTB domain lacking the C terminus of Dab1 increased secreted APP and APP CTF (Fig. 2*C*). The PTB domain lacking the C terminus of Dab1 expression slightly increased cell APP levels (Fig. 2*C*). We also transfected COS7 cells with APP and the PTB deletion construct of Dab1, and we measured the amount of secreted APP and APP CTF. The PTB deletion construct of Dab1 did not affect levels of any APP proteolytic product as follows: secreted APP, APP α-CTF, or APP β-CTF (Fig. 2*D*).

To test the nature of soluble and cell-associated protein bands, we transfected APP and measured the cell-associated APP and secreted APP. We found that the band for cell-associated APP is slightly higher than that for sAPP (Fig. 2*F*). Finally, because Dab1 affected APP processing, we examined whether it affected Aβ production in COS7 cells. Full-length Dab1 significantly decreased secreted Aβ40 levels (by 68%) and Aβ42 levels (by 44%) (Fig. 2*E*).

APP-Dab1 Interactions Do Not Alter APP or Dab1 Phosphorylation—Tyrosine phosphorylation of Dab1 is important for its signal-transducing effects in development (20). To test

whether the interaction of Dab1 with APP affects Dab1 phosphorylation, we expressed APP with Dab1 in COS7 cells as above. We included a form of Dab1 in which its five tyrosine residues were replaced with phenylalanine (5F) as a negative control. As a positive control, we also included a constitutively active form of the kinase Fyn, which phosphorylates both Dab1 and APP (21). We immunoprecipitated Dab1 and probed for phosphotyrosine. Both full-length and mutant 5F Dab1 did not show anti-phosphotyrosine immunoreactivity (Fig. 3*A*). However, co-transfection with Fyn significantly increased anti-phosphotyrosine Dab1 immunoreactivity (Fig. 3*A*, *1st panel*). We also performed the reverse experiment, immunoprecipitating with anti-phosphotyrosine antibody and probing with anti-Dab1 antibody (Fig. 3*A*, *2nd panel*). Co-transfection with Fyn significantly increased anti-Dab1 immunoreactivity, but no phospho-Dab1 was observed in the presence of APP alone. To measure the expression levels of Dab1, we immunoprecipitated with an anti-Dab1 antibody, and we probed with a second anti-Dab1 antibody (Fig. 3*A*, *3rd panel*). Strong Dab1 and Dab1 5F immunoreactivity were observed in all the transfected samples. Finally, we measured Fyn and APP levels in transfected cells via immunoblot to demonstrate their expression (Fig. 3*A*, *4th* and *5th panels*).

We similarly asked whether the interaction of Dab1 with APP affected APP phosphorylation, using the same cells. We immunoprecipitated APP with an anti-c1/6.1 antibody, and probed for phosphotyrosine. COS7 cells transfected with Fyn had measurable levels of phospho-APP (Fig. 3*B*, *5th lane, 1st panel*), but expression of full-length Dab1 or Dab1 5F constructs did not result in phospho-APP. Levels of full-length Dab1 and Dab1 5F were similar and levels of APP were consistent across transfections (Fig. 3*B*, *lower panels*). These results suggest that unphosphorylated Dab1 is capable of binding APP and altering its processing without affecting its tyrosine phosphorylation.

Dab1 Affects APP and ApoEr2 Processing via Their NPXY Motifs—To test whether the NPTY motif of APP was important in the effect of Dab1 on processing of APP (as expected), we used a construct of APP where the NPTY sequence was changed to APTY. We transfected COS7 cells with full-length Dab1 and wild-type or mutant APP, and we measured the amount of APP CTF and secreted APP. Levels of cellular APP, APP CTF, and sAPP were similar in cells transfected with wild-type APP *versus* mutant APP (Fig. 4*A*, *1st* and *3rd lanes*). Dab1 co-expression increased APP CTF and sAPP from wild-type APP (Fig. 4*A*, *2nd lane*). However, Dab1 co-expression did not alter levels of mutant APP CTF or sAPP (Fig. 4*A*, *4th lane*). Levels of full-length cell-associated APP were not altered by Dab1 co-expression (Fig. 4*A*, *middle panel*).

To similarly test whether the NPVY motif of apoEr2 was important in the effect of Dab1 on processing of apoEr2, we generated a construct of apoEr2 where the NPVY sequence was changed to APVY. We transfected COS7 cells with full-length Dab1 and wild-type or mutant apoEr2, and we measured the amount of apoEr2 CTF and secreted apoEr2. Levels of cellular apoEr2, apoEr2 CTF, and sapoEr2 were similar in cells transfected with wild-type apoEr2 *versus* mutant apoEr2 (Fig. 4*B*, *1st* and *3rd lanes*). Dab1 co-expression increased apoEr2 CTF and sapoEr2 from wild-type apoEr2 (Fig. 4*B*, *2nd lane*), but Dab1 co-expression did not alter the levels of mutant apoEr2 CTF or sapoEr2 (Fig. 4*B*, *4th lane*). Levels of total cell-associated apoEr2 were not altered by Dab1 co-expression. Thus, the

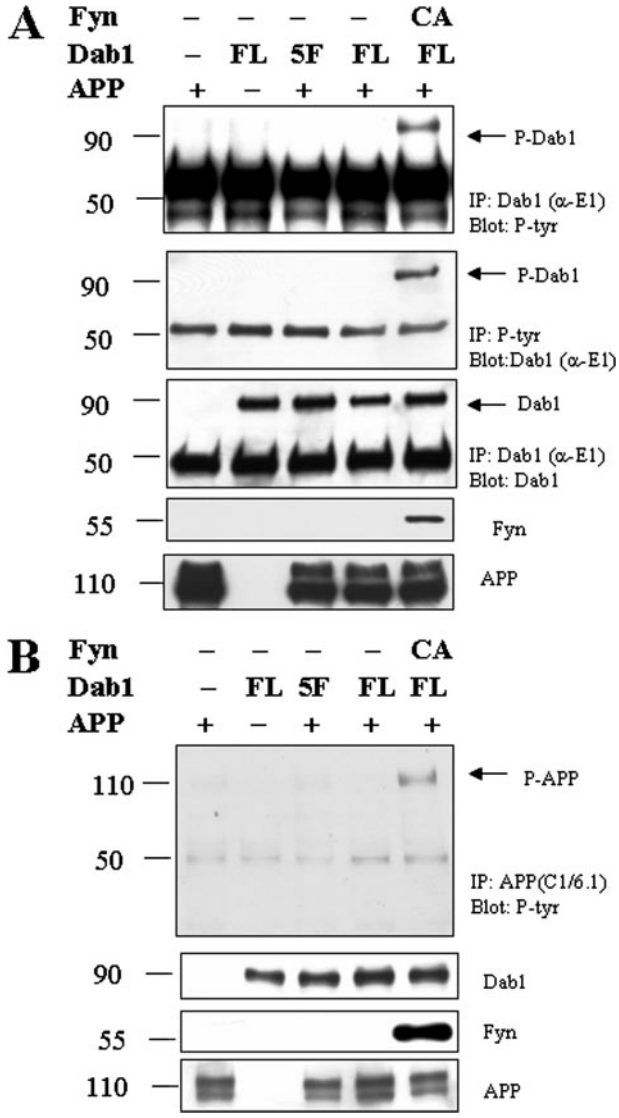

FIGURE 3. **The APP-Dab1 interaction does not affect phosphorylation of APP or Dab1.** *A*, COS7 cells were transiently transfected with APP and vector, APP and Dab1 5F (lacking tyrosine residues), APP and Dab1, or APP, Dab1, and a constitutively active form of Fyn kinase (*CA*). Cell lysates (200 μg) were immunoprecipitated (*IP*) with anti-Dab1, and the precipitate was probed with an anti-phosphotyrosine antibody. Both full-length and mutant 5F Dab1 did not show anti-phosphotyrosine immunoreactivity. However, co-transfection with Fyn-CA significantly increased anti-phosphotyrosine immunoreactivity (*upper panel*). Cell lysates (200 μg) were immunoprecipitated with phosphotyrosine, and the precipitate was probed with an anti-Dab1. Both full-length and mutant 5F Dab1 did not show anti-Dab1 immunoreactivity. Co-transfection with Fyn-CA significantly increased anti-Dab1 immunoreactivity (*2nd panel*). Cell lysates (200 μg) were immunoprecipitated with Dab1, and the precipitate was probed with an anti-Dab1 (*3rd panel*). Lysates from co-transfected cells (20 μg) were probed for APP or Fyn to demonstrate expression (the *4th* and *5th panels*). *B*, COS7 cells were transiently transfected with APP and vector, APP and Dab1 5F, APP and full-length Dab1, or APP, Dab1, and Fyn-CA. Cell lysates (200 μg) were immunoprecipitated with anti-c1/6.1, and the precipitate was probed with anti-phosphotyrosine. No increase in phospho-APP was observed in the presence of Dab1 5F or full-length Dab1, but co-transfection with Fyn-CA significantly increased phospho-APP. Lysates from co-transfected cells (20 ug) were probed for Dab1, Fyn, or APP to demonstrate expression levels (*lower panels*).

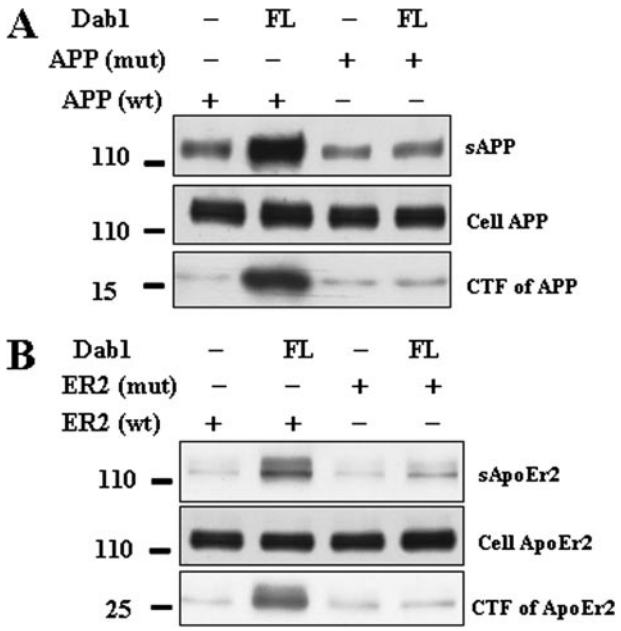

FIGURE 4. **The NP*X*Y motifs of APP and apoEr2 mediate Dab1 effects.** *A,* COS7 cells were transfected with full length Dab1 (FL) and wild-type APP (*1st* and *2nd lanes*) or an APP APTY mutant (*3rd* and *4th lanes*). Conditioned media were analyzed for secreted forms of APP (6E10); cell lysates were analyzed for α-CTF of APP (c1/6.1). Dab1 increased wild-type APP processing (*1st* and *2nd lanes*) but not processing of the APP APTY mutant (*3rd* and *4th lanes*). *B,* COS7 cells were transfected with Dab1 and wild-type apoEr2 (*1st* and *2nd lanes*) or an apoEr2 APVY mutant (*3rd* and *4th lanes*). Secreted apoEr2 was measured in conditioned media with antibody 5810, and apoEr2 CTFs were detected from cell lysates with anti-HA antibody. Dab1 increased wild-type apoEr2 processing (*1st* and *2nd lanes*) but not processing of the apoEr2 APVY mutant (*3rd* and *4th lanes*).

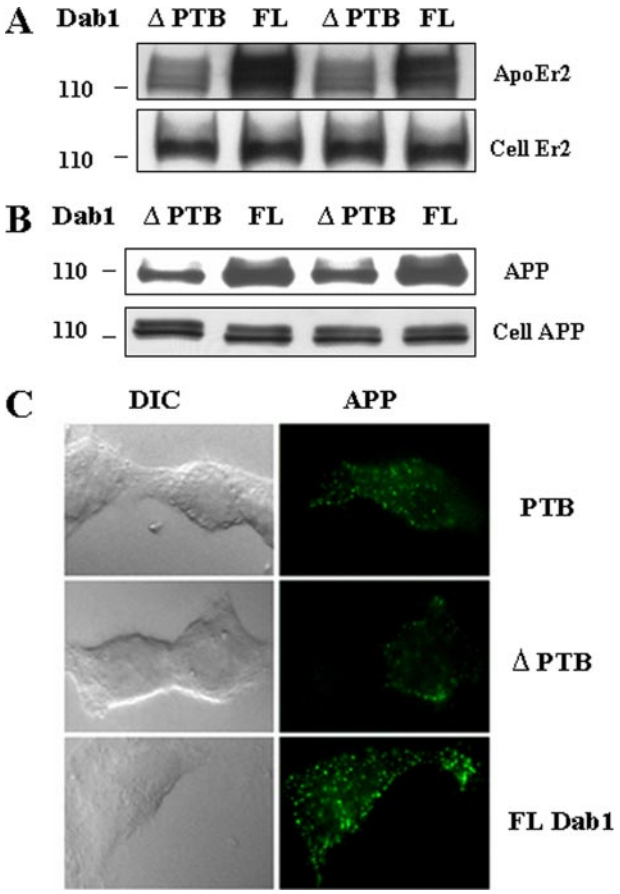

FIGURE 5. **Dab1 increases cell surface apoEr2 and APP.** *A,* COS7 cells were transfected with full-length apoEr2 and full-length Dab1 or Dab1 ΔPTB. Cell surface proteins were biotin-labeled, isolated with avidin beads, and immunoblotted with 5810 for apoEr2. Full-length Dab1 increased surface levels of apoEr2 (*upper blot*). Immunoblots of cell lysates showed similar levels of total apoEr2 (*lower blot*). *B,* COS7 cells were transfected with full-length APP and these Dab1 constructs. Cell surface proteins were isolated and immunoblotted with 6E10 for APP. Full-length Dab1 increased surface levels of APP (*upper blot*). Immunoblots of cell lysates showed similar levels of total APP (*lower blot*). *C,* HEK293 cells were transfected with full-length APP and Dab1 constructs. Cells were incubated with 22C11 (for APP) and then incubated with Alexa Fluor 488 anti-mouse antibody (*green color*). Cells were imaged with DIC 3 (*left panel*). Dab1 PTB and full-length Dab1 increased surface staining levels of APP (*right panel*).

NP*X*Y sequences were important to the effects of Dab1 on both APP and apoEr2 processing.

Dab1 Increases Levels of Cell Surface APP and ApoEr2—A recent study has shown that Dab1 regulates both cell surface expression and internalization of apoEr2 and VLDLr (22). Thus, the effects of Dab1 on APP or apoEr2 cleavage could be caused by changes in APP and apoEr2 trafficking. To examine the effect of Dab1 on cell surface levels of APP or apoEr2, we co-transfected COS7 cells with APP or apoEr2 and the various constructs of Dab1 (Fig. 1*A*). We measured cell surface proteins by biotin-labeling live cells and isolated biotin-labeled proteins from lysates with avidin beads and immunoblotting apoEr2 or APP. Levels of cell surface apoEr2 and APP were increased by co-expression of full-length Dab1 (Fig. 5, *A* and *B*, *upper panels*) compared with Dab1 without the PTB domain (which did not affect APP or apoEr2 processing (Figs. 1 and 2)). Quantification of the Western blots showed a 340% increase in apoEr2 and a 270% increase in APP in the presence of full-length Dab1 ($p < 0.01$). Levels of total cell-associated APP or apoEr2 were similar among the transfected cells (Fig. 5, *A* and *B*, *lower panels*). We also found that levels of cell surface APP or apoEr2 were increased by constructs containing only the PTB domain of Dab1 (data not shown).

As another measure of cell surface levels, we immunostained live cells. HEK293 cells were transfected with APP and either the PTB domain of Dab1, the PTB deletion construct of Dab1, or the full-length Dab1. We found that the PTB domain and full-length Dab1, which interact with APP, increased cell surface expression of APP compared with the PTB truncated Dab1, which does not interact with APP (Fig. 5*C*). Thus, both assays demonstrated that Dab1 constructs containing the PTB domain increased surface levels of APP.

Dab1 Alters the Fe65-APP Interaction—Other adaptor proteins bind the APP intracellular domain, potentially affecting the interaction with Dab1. We asked whether Dab1 affects the interaction of Fe65 with the cytoplasmic product of γ-secretase cleavage of APP CTF, the APP intracellular domain (AICD). We used an Fe65-dependent APP luciferase transactivation assay to assess AICD in the presence of a Dab1 deletion mutant or full-length Dab1. HEK293 cells were co-transfected with APP-Gal4, Fe65, a luciferase reporter construct (G5E1B-Luc), and Dab1 constructs. Full-length Dab1 resulted in a 75% reduction in Fe65-dependent transactivation. However, the deletion mutant of Dab1 without the PTB domain did not affect the production of AICD (Fig. 6). Levels of Fe65 were similar among the transfected cells (Fig. 6, *lower panel*).

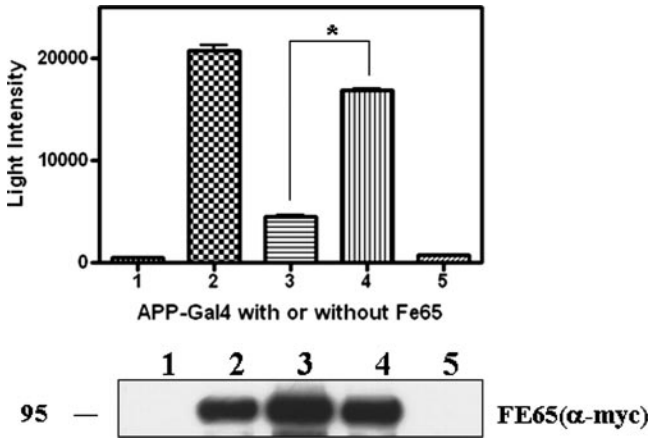

FIGURE 6. **Dab1 and Fe65 alter AICD signaling.** HEK293 cells were transfected with APP-Gal4, Fe65, and Dab1 constructs as shown in Fig. 1. Transfections were as follows: *lane 1*, G5E1B-Luc, APP-Gal4; *lane 2*, G5E1B-Luc, APP-Gal4 and Fe65; *lane 3*, G5E1B-Luc, APP-Gal4, full-length Dab1, and Fe65; *lane 4*, G5E1B-Luc, APP-Gal4, Dab1 without PTB domain, and Fe65; *lane 5*, G5E1B-Luc, APP-Gal4, and full-length Dab1. Full-length Dab1, but not Dab1 lacking the PTB domain, decreased Fe65-mediated APP luciferase transactivation (*, $p < 0.01$). Lysates from co-transfected cells (20 μg) were probed for FE65 to demonstrate expression levels (*lower panel*).

Reelin Increases the Interaction between APP and Dab1 or ApoEr2 and Dab1—Dab1 is necessary for the regulation of neuronal positioning in the developing brain (12), which is affected by binding of the extracellular matrix molecule Reelin to the neuronal receptors apoEr2 and VLDLr (23). We tested whether Reelin altered the interaction between Dab1 and apoEr2. We co-expressed full-length apoEr2 with Dab1, and we treated cells with control or Reelin-containing media for 24 h. Reelin significantly increased co-immunoprecipitation of Dab1 with apoEr2 compared with control medium (Fig. 7*A*). Quantification of Western blots demonstrated that apoEr2/Dab1 co-precipitation was increased by 245% after Reelin treatment. The levels of apoEr2 and Dab1 did not vary across conditions (Fig. 7*A*). We tested whether Reelin had similar effects on endogenous apoEr2 and Dab1 in primary neurons. We immunoprecipitated Dab1, and probed for apoEr2 Dab1 with 5810. As expected, endogenous Dab1 co-precipitated with apoEr2 (Fig. 7*B*). Reelin treatment increased this co-precipitation but did not affect total levels of apoEr2 or Dab1 (Fig. 7*B*). Quantification of Western blots demonstrated that apoEr2/Dab1 co-precipitation was increased by 87% after Reelin treatment. Interestingly, this increase was observed after only 30 min of Reelin treatment.

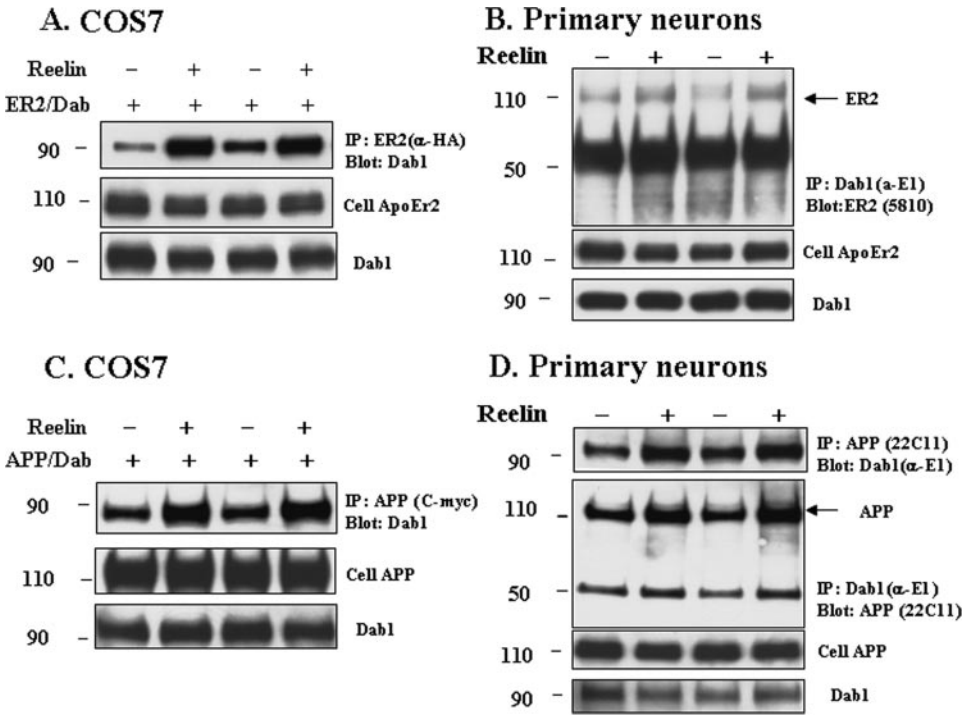

FIGURE 7. **Reelin increased co-immunoprecipitation of apoEr2 with Dab1 and APP with Dab1.** *A*, COS7 cells transfected with apoEr2 and Dab1 were treated with control media (−) or conditioned media containing Reelin (+) for 24 h. Cell lysates (200 μg) were immunoprecipitated (*IP*) with anti-HA antibody and probed with anti-Dab1 antibody. Reelin increased co-immunoprecipitation of apoEr2 and Dab1. *B*, primary neurons were treated with Reelin (*2nd* and *4th lanes*) or control (*1st* and *3rd lanes*) media for 30 min. Primary neuronal proteins (1 mg) were immunoprecipitated with α-Dab1 and probed with 5810. ApoEr2 and Dab1 co-precipitated, and this interaction was increased by Reelin. Immunoblot of cell lysates showed similar levels of apoEr2 and Dab1 (*middle* and *lower panels*). *C*, COS7 cells transfected with APP and Dab1 were treated with control media (−) or conditioned media containing Reelin (+) for 24 h. Cell lysates (200 μg) were immunoprecipitated with anti-C-Myc antibody and probed with anti-Dab1 antibody. Reelin increased co-immunoprecipitation of APP and Dab1. *D*, primary neurons were treated with Reelin (*2nd* and *4th lanes*) or control (*1st* and *3rd lanes*) media for 30 min. Primary neuronal proteins (1 mg) were immunoprecipitated with 22C11 and probed with anti-Dab1. APP and Dab1 co-precipitated, and this interaction was increased by Reelin. Conversely, primary neuronal protein (1 mg) was immunoprecipitated with Dab1 and probed with 22C11 (for APP). Again, APP and Dab1 co-precipitated, and this interaction was increased by Reelin. Immunoblot of cell lysates showed similar levels of APP and Dab1 (*lower panels*).

We similarly asked whether the APP and Dab1 interaction was increased by Reelin. We co-expressed full-length APP with Dab1 and treated cells with control or Reelin-containing media for 24 h. As with apoEr2, Reelin significantly increased co-precipitation of Dab1 with APP (Fig. 7*C*). The levels of APP and Dab1 did not vary across conditions (Fig. 7*C*). Quantification of Western blots demonstrated that APP/Dab1 co-precipitation was increased by 124% after Reelin treatment. It is unknown how the extracellular Reelin protein affected the intracellular interactions between Dab1 and apoEr2 or APP.

We tested whether Reelin affected the interaction between endogenous APP and Dab1 in primary neurons. We immunoprecipitated APP with antibody 22C11 and probed for Dab1 with an anti-Dab1 antibody. As expected, endogenous Dab1 co-precipitated with APP (Fig. 7*D*). Reelin treatment increased this co-precipitation but did not affect total levels of APP or Dab1 (Fig. 7*D*). We also immunoprecipitated Dab1 with an anti-Dab1 antibody and probed for APP with 22C11. Endogenous APP consistently co-precipitated with Dab1, and Reelin treat-

ment increased this co-precipitation (but did not affect total levels of APP or Dab1) (Fig. 7*D*). Quantification of Western blots demonstrated that Reelin treatment increased the APP/Dab1 interaction by 97%.

Reelin Affects APP and ApoEr2 Processing—The data above demonstrate that Reelin increased the interaction between apoEr2 and Dab1 and between APP and Dab1. Therefore, we hypothesized that Reelin might also increase processing of apoEr2 and APP, similar to our findings with Dab1 (Figs. 1 and 2). To address this hypothesis, we treated COS7 cells transfected with apoEr2 and Dab1 with Reelin-containing medium. Compared with control medium, Reelin treatment increased the levels of secreted apoEr2 and apoEr2 CTF, without affecting total levels of apoEr2 (Fig. 8*A*).

In order to assess whether Reelin affected APP processing in the presence of Dab1, we treated COS7 cells transfected with APP and Dab1 with Reelin-containing medium. We found that Reelin also increased the levels of secreted APP and APP CTF, without affecting total levels of APP (Fig. 8*B*). We also treated COS7 cells transfected with APP and the PTB domain of Dab1 with Reelin-containing medium. We found that Reelin increased the levels of secreted APP and APP CTF (Fig. 8*C*). Levels of full-length APP also appeared higher in the Dab1 PTB-containing cells.

Because Reelin affected APP processing, we examined whether it affected Aβ production. COS7 cells transfected with APP and Dab1 were treated with Reelin-containing medium. We observed that Reelin significantly decreased secreted levels of both Aβ40 (by 72%) and Aβ42 (by 60%) (Fig. 9*A*). We also tested whether the PTB domain of Dab1 affected Aβ production. COS 7 cells transfected with APP and the PTB domain of Dab1 were treated with Reelin-containing medium. We observed that Dab1 PTB decreased Aβ40 (by 61%), but Reelin treatment did not lead to a further significant reduction in Aβ (data not shown).

In addition, we examined whether Reelin affected the processing of APP in neurons from APPsw-transgenic mice. Primary cortical neurons were isolated from APPsw-tg and wild-type littermates. After 18 h of Reelin treatment, the levels of secreted Aβ40 collected from the culture media of APPsw-tg neurons were significantly lowered (by 32%) compared with littermate controls (Fig. 9*B*).

Finally, we assessed the levels of endogenous mouse Aβ in primary neurons from wild-type, $DAB^{+/-}$, or $DAB^{-/-}$ embryos (Fig. 9, *C* and D). The DAB1 knock-out phenotype was confirmed by PCR (Fig. 9*C*). Primary neurons from $DAB^{-/-}$ mice had significantly higher Aβ40 and Aβ42 levels compared with neurons from littermate controls, supporting the hypothesis that Dab1 is important for reducing Aβ production (Fig. 9, *C* and *D*).

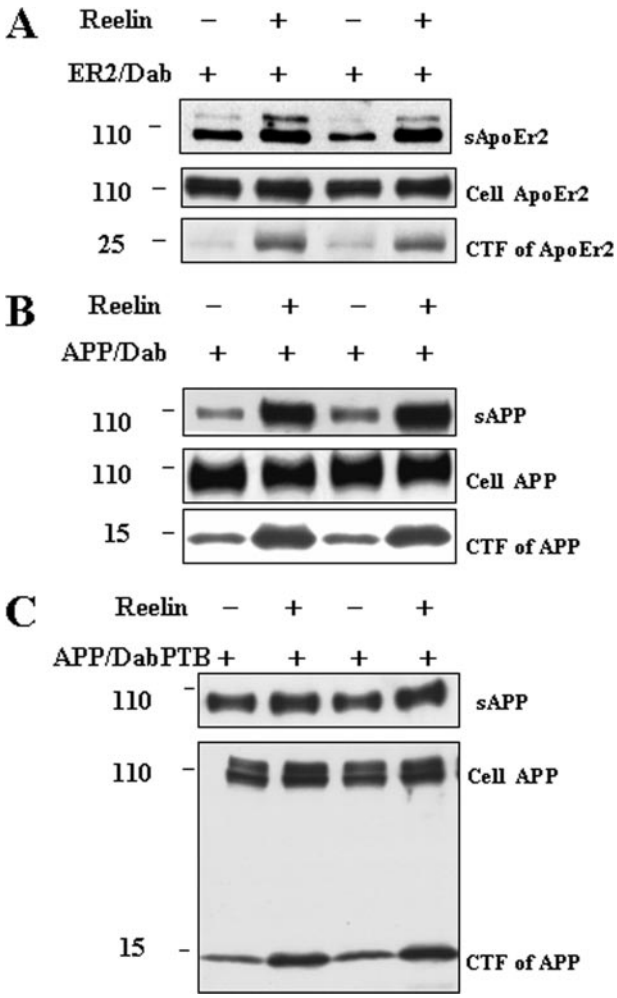

FIGURE 8. **Reelin alters APP and apoEr2 processing.** *A*, COS7 cells were transiently transfected with apoEr2 and full-length Dab1. After transfection, cells were treated with control or Reelin-containing medium. Secreted apoEr2 was measured in conditioned media with antibody 5810, and apoEr2 CTFs were detected from cell lysates with anti-HA antibody. Reelin treatment increased secreted apoEr2 and apoEr2 CTF in the presence of Dab1. *B*, COS7 cells were transiently transfected with APP and Dab1 and treated with control or Reelin-containing medium. Secreted APP was measured in conditioned media with antibody 6E10, and APP CTF was detected from cell lysates with C1/6.1 antibody. Reelin treatment increased secreted APP and APP CTF in the presence of Dab1. *C*, COS7 cells were transiently transfected with APP and Dab1 PTB and treated with control or Reelin-containing medium for 24 h. Secreted APP was measured in conditioned media with antibody 6E10, and APP CTF was detected from cell lysates with anti-C1/6.1 antibody. Reelin treatment increased secreted APP and APP CTF in the presence of the PTB domain of Dab1.

DISCUSSION

Dab1 is an adaptor protein important in proper neuronal migration during development, because of its binding to the cytoplasmic domain of apoE receptors. Dab1 also binds to the cytoplasmic domain of APP, where it may play a role in the functions of APP. Other adaptor proteins affect trafficking and processing of APP or apoEr2, such as Fe65 (3) and Mint/X11 (7). In this study, we found that Dab1 resulted in increased cell surface APP and apoEr2 (Fig. 5) and increased cleavage of APP and apoEr2 to release secreted extracellular domains and membrane-bound CTFs (Figs. 1 and 2). These effects depended on the PTB domain of Dab1. The PTB domain of Dab1 is also important for localizing the protein at the cell membrane by binding to phosphoinositides (24), an interaction that is independent of Dab1 binding to APP or apoEr2 (25, 26). The effects of Dab1 on APP and apoEr2 could result from a competition between Dab1 and the other adaptor proteins binding to APP and apoEr2, preventing them from affecting trafficking and processing.

Overexpression of Dab1 led to increased α-cleavage of APP and decreased β-cleavage and a decrease in the levels of Aβ40

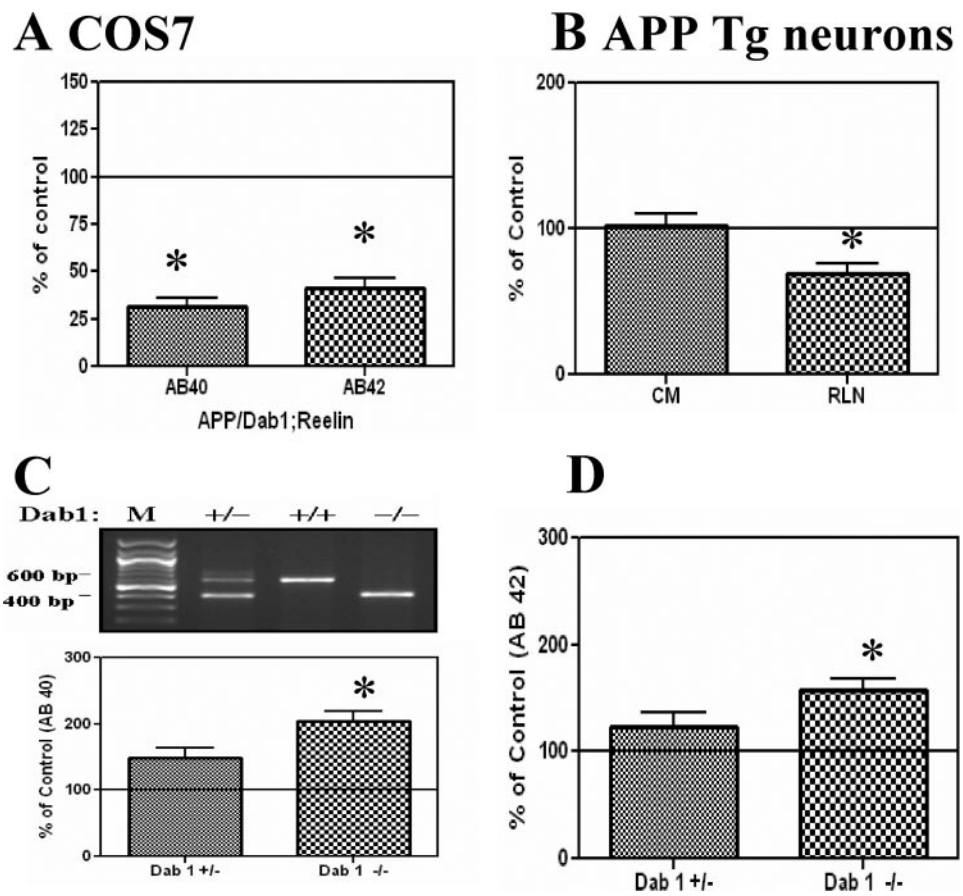

FIGURE 9. **Reelin decreases Aβ.** *A,* COS7 cells were transiently transfected with APP and Dab1. After transfection, cells were treated with control or Reelin-containing medium. Aβ40 and Aβ42 levels in the conditioned media were determined by ELISA. Reelin significantly decreased secreted Aβ40 levels (by 72%, $p < 0.05$), and Aβ42 levels (by 60%, $p < 0.05$). *B,* primary cortical neurons were treated with either Reelin-conditioned medium or control medium for 18 h. Supernatants were collected, and levels of secreted Aβ were determined by ELISA. Levels of secreted Aβ40 from Reelin-stimulated cells were significantly decreased by 32% (*, Student's *t* test: $p < 0.05$). *C,* the DAB1 knock-out phenotype was confirmed by PCR (*upper panel*): wild-type, 600 bp; $DAB^{+/-}$, both 600 bp and 400 bp; $DAB^{-/-}$, 400 bp (*M*, DNA markers). Aβ40 levels were measured in primary cortical neurons and compared with levels from wild-type neurons (set to 100%). Primary neurons from $DAB^{-/-}$ had significantly increased secreted Aβ40 levels (by 101%). *D,* conditioned media from wild-type, $DAB^{+/-}$, $DAB^{-/-}$ primary neurons were measured for secreted Aβ42. Again, primary neurons from $DAB^{-/-}$ had significantly increased Aβ42 levels (by 77%).

and Aβ42 (Fig. 2). No changes in total APP or apoEr2 were observed, suggesting that only a small percentage of APP is cleaved under these conditions. Knock-out of endogenous Dab1 in primary neurons resulted in increased levels of Aβ40 and Aβ42 (Fig. 8).

A recent study also examined the effect of Dab1 on APP processing, using transfected HEK cells (27). They similarly observed that Dab1 increased surface APP and APP α-cleavage. However, in contrast to our results, they observed increased APP β-CTF and Aβ levels (27) in the presence of Dab1. Cell-line specific differences have been observed for the effects of FE65 on APP processing (3, 5, 28), an observation we have confirmed in COS7 and Chinese hamster ovary cells (29). Cytoplasmic adaptor proteins will affect trafficking and processing of full-length APP as well as APP CTFs, and thus there may be differences in cell lines dependent on which adaptor proteins are endogenously present. In support of this model, we have found that Dab1 competes with Fe65 for interaction with AICD (Fig. 6). Our data in primary neurons and mouse brains suggest that the effect of Dab1 in neurons is similar to what we observed in transfected COS7 cells.

Adaptor proteins that interact with cytoplasmic NP*X*Y sequences differ in their preference for binding sequences that have or lack phosphorylation of the tyrosine residue. Disruption of the NP*X*Y motif in APP or apoEr2 prevented the effects of Dab1 on trafficking and processing (Fig. 4). Immunoprecipitation of Dab1 did not co-precipitate a phosphorylated version of APP (Fig. 3), consistent with findings that Dab1 binds preferentially to nonphosphorylated NP*X*Y sequences (13, 20). Thus, phosphorylation of APP or apoEr2 may regulate whether these molecules interact with Dab1 or with other adaptor proteins.

APP and apoEr2 are linked extracellularly by binding different domains of F-spondin (30); the presence of F-spondin increases the interaction between APP and apoEr2 (31). As extracellular interactions cluster the proteins together in the membrane, the intracellular domains interact, and the adaptor proteins bound to the intracellular domains interact. Because Dab1 interacts with both APP and apoEr2 through its PTB domain, these proteins may compete for binding a Dab1 molecule. In contrast, the adaptor protein FE65 binds APP and apoEr2 through two separate PTB domains, forming a tripartite complex (29), similar to a complex demonstrated for APP, FE65, and another apoE receptor, low density lipoprotein receptor-related protein (32). Dab1 and FE65 both bind the NP*X*Y sequences of APP and apoEr2, and thus Dab1 could prevent the FE65 intracellular link between APP and apoEr2.

It is unknown how Dab1 transduces the signal from extracellular ligands binding to receptors such as apoEr2. Dab1 is phosphorylated when Reelin binds to apoEr2, potentially affecting downstream functions. In this study, we did not observe any changes in Dab1 phosphorylation in the presence of APP or apoEr2 (Fig. 3) or after treatment of COS7 cells with Reelin (data not shown). We did observe that Reelin increased the co-immunoprecipitation of Dab1 with apoEr2 or APP (Fig. 7) and affected their trafficking and processing (Fig. 8). Thus, we hypothesize that binding of proteins to the extracellular domains of APP or apoEr2 may alter the trafficking of these transmembrane proteins and favor the binding of Dab1 to their cytoplasmic domains. Reelin alone reduced Aβ levels in the presence of Dab1 (Fig. 9) but did not affect Aβ levels in the

presence of only the PTB1 domain of Dab1, supporting this hypothesis. We are currently examining whether binding of other ligands of APP or apoEr2 (such as F-spondin or apoE isoforms) affects the interaction of Dab1 with these cell surface proteins.

Many of the interacting molecules examined in this study are important for migration of neurons in the cortex. Reelin and Dab1 knock-out mice show neuronal migration defects (33), similar to deficits seen in apoEr2 and VLDLr knock-out mice (12). Disrupting APP and its two mammalian homologues, APLP1 and APLP2, is generally lethal, but the rare surviving embryos show cortical dysplasias (34), suggesting that the APP protein family members may also have important functions in neuronal migration. Finally, an Fe65/Fe65L1 double knock-out mouse model also demonstrated cortical dysplasias and axonal projection deficiencies (35). Further research into this complex of proteins may provide insight into the mechanisms necessary for neuronal migration.

The data described in this study demonstrate an interaction between Dab1 and APP and apoEr2, altering processing of both of these transmembrane proteins. These effects are promoted by the presence of Reelin, suggesting that extracellular factors alter intracellular interactions important for protein trafficking and processing. These findings underscore a potentially important role for APP and associated proteins in neuronal migration.

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