Published in final edited form as:

FASEB J. 2014 January ; 28(1): 337–349. doi:10.1096/fj.13-232694.

FE65 interacts with ADP-ribosylation factor 6 to promote neurite outgrowth

Hei Nga Maggie Cheung^{*}, Charlotte Dunbar[†], Gábor M. Mórotz[†], Wai Hang Cheng^{*}, Ho Yin Edwin Chan^{*}, Christopher C. J. Miller[†], and Kwok-Fai Lau^{*,1}

^{*}School of Life Sciences, Chinese University of Hong Kong, Shatin, Hong Kong, China

[†]King's College London Centre for Neurodegeneration Research, Department of Neuroscience, King's College London Institute of Psychiatry, London, UK

Abstract

FE65 is an adaptor protein that binds to the amyloid precursor protein (APP). As such, FE65 has been implicated in the pathogenesis of Alzheimer's disease. In addition, evidence suggests that FE65 is involved in brain development. It is generally believed that FE65 participates in these processes by recruiting various interacting partners to form functional complexes. Here, we show that *via* its first phosphotyrosine binding (PTB) domain, FE65 binds to the small GTPase ADP-ribosylation factor 6 (ARF6). FE65 preferentially binds to ARF6-GDP, and they colocalize in neuronal growth cones. Interestingly, FE65 stimulates the activation of both ARF6 and its downstream GTPase Rac1, a regulator of actin dynamics, and functions in growth cones to stimulate neurite outgrowth. We show that transfection of FE65 and/or ARF6 promotes whereas small interfering RNA knockdown of FE65 or ARF6 inhibits neurite outgrowth in cultured neurons as compared to the mock-transfected control cells. Moreover, knockdown of ARF6 attenuates FE65 stimulation of neurite outgrowth and defective neurite outgrowth seen in FE65-deficient neurons is partially corrected by ARF6 overexpression. Notably, the stimulatory effect of FE65 and ARF6 on neurite outgrowth is abrogated either by dominant-negative Rac1 or knockdown of Rac1. Thus, we identify FE65 as a novel regulator of neurite outgrowth *via* controlling ARF6-Rac1 signaling.

Keywords

ARF6; amyloid- β ; A4 precursor protein-binding family B member 1; neurons; Rac1

FE65 is an adaptor protein that interacts with the intracellular C terminus of the Alzheimer's disease amyloid precursor protein (APP; ref. 1–3). The interaction between FE65 and APP can influence APP processing and production of amyloid- β (A β) peptide that is deposited in the brains of Alzheimer's disease patients (4–8). In addition, FE65 and APP participate in nuclear signaling (9, 10) and DNA repair after damage (11, 12) and these functions have also been linked to Alzheimer's disease (for reviews, see refs. 13–15).

¹Correspondence: School of Life Sciences, Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR, China. kflau@cuhk.edu.hk.

This article includes supplemental data. Please visit <http://www.fasebj.org> to obtain this information.

However, along with these nuclear functions, FE65 is also believed to have cytoplasmic roles in neurons and in particular in neurodevelopmental processes and synaptic function. Evidence to support this notion comes from a number of findings. First, FE65 is developmentally regulated in the brain and has been linked to neurogenesis (16, 17). Also, FE65-knockout mice display defective brain development (5). Second, FE65 regulates actin-based membrane motility and localizes in actin-rich mobile structures within the growth cones (18, 19). Finally, learning and memory deficits have been reported in FE65-knockout mice, and these are linked to synaptic changes (8, 20). However, the precise mechanisms by which FE65 regulates neuronal development and synaptic function are not properly understood.

Here, we show that FE65 binds to ADP-ribosylation factor 6 (ARF6). ARF6 is a ubiquitously expressed Ras superfamily GTPase that is involved in both endocytic membrane trafficking and actin cytoskeletal rearrangements. These functions are regulated by cycling of ARF6 between GTP (active) and GDP (inactive) states that are modulated *via* the actions of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (for review, see ref. 21). In the nervous system, ARF6 has been shown to regulate early neuronal morphogenesis (for review, see ref. 22) and axon development (23, 24). The effect of ARF6 on neural development involves, at least in part, Rac1, which is a regulator of actin dynamics (for reviews, see refs. 25, 26). In this report, we characterize FE65-ARF6 interaction and demonstrate that FE65 and ARF6 promote neurite outgrowth and regulate the activation of Rac1.

MATERIALS AND METHODS

Yeast 2-hybrid system

Yeast 2-hybrid screens were performed essentially as described previously (27). Briefly, sequence encoding the human FE65 phosphotyrosine binding (PTB) 1 + 2 domains (aa 361–676 of FE65) was subcloned into the yeast “bait” pGBKT7 vector and then transformed into yeast Y2H Gold. To perform the library screen, the bait-containing yeast Y2H Gold was mated with yeast Y187 pretransformed with a human brain cDNA library (Clontech, Mountain View, CA, USA). After selection, vigorously growing clones were subjected to freeze-fracture β -galactosidase assays. Candidate library pACT2 plasmids were rescued by transforming into *Escherichia coli* DH5 α , and the brain library cDNA inserts were then sequenced.

Cell culture and transfection

Chinese hamster ovary (CHO) and SH-SY5Y cells were cultured as described previously (27, 28). Primary rat cortical neurons were dissected from E18 embryos and grown on glass coverslips coated with poly-D-lysine in Neurobasal medium with B27 supplement (Life Technologies, Grand Island, NY, USA).

For plasmid transfection, CHO cells were transfected with FuGene 6 (Roche, Perzberg, Germany), and SH-SY5Y and rat cortical neurons were transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturers' instructions and as described

previously (29). All siRNAs were obtained from Dharmacon ThermoScientific (Rockford, IL, USA). For CHO and SHSY5Y cells, siRNAs were transfected using Lipofectamine RNAiMAX (Life Technologies). For cultured neurons, cells were incubated with Accell siRNAs previously described (27, 29). For cytochalasin D (CytoD; Life Technologies) treatment, neurons were incubated with 0.25 µg/ml CytoD (in DMSO) for 24 h.

Plasmids

A mammalian expression vector of glutathione *S* transferase (GST) was prepared by cloning GST cDNA into pCI-neo (Promega, Madison, WI, USA) to form pCIneo-GST. The GST fusion protein constructs of pCIneo-GST-FE65 WW, pCIneo-GST-FE65 PTB1, and pCIneo-GST-FE65 PTB2 were made by subcloning of the FE65 corresponding cDNAs (WW domain, aa 248–290; PTB1 domain, aa 361–514; and PTB2 domain, aa 531–676) into pCIneo-GST, respectively. The GST fusion protein constructs of pCIneo-GST-ARF6, pCIneo-GST-ARF6 1–80, pCIneo-GST-ARF6 28–175, pCIneo-GST-ARF6 48–175, and pCIneo-GST-ARF6 73–175 were made by subcloning of the ARF6 corresponding cDNAs into pCIneo-GST, respectively. Mammalian expression constructs for wild-type FE65, myc-tagged FE65, myc-tagged FE65 PTB1, and APP were as described previously (9, 10, 30). Wild-type Rac1 and N17Rac1 were as described previously (31). V12Rac1 mutant was generated by using QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Myc/His- and GST-tagged wild-type ARF6 constructs were generated by subcloning the full-length ARF6 cDNA into pcDNA3.1/myc-His vectors (Life Technologies) and pGEX-6P-1 (GE Healthcare Biosciences, Pittsburgh, PA, USA), respectively. Myc/His-tagged mutant ARF6 T27N and Q67L were generated by site-directed mutagenesis.

Antibodies

Mouse (9B11) and rabbit (71D10) anti-myc antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit anti- α -tubulin and mouse anti- α -tubulin (DM1A) were from Abcam (Cambridge, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Anti-polyHistidine (HIS-1) and anti-GST were purchased from Sigma. Rabbit anti- α -tubulin was from Abcam. Anti-Rac1 (23A8) was obtained from Millipore (Billerica, MA, USA). Rat polyclonal antibody against ARF6 (AR3) was created by immunization of a rat with GST-ARF6 fusion protein. Anti-ARF6 antibodies 3A-1 and 6ARF01 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Millipore, respectively. Rabbit anti-FE65 was as described previously (32); goat anti-FE65 (E20) was obtained from Santa Cruz Biotechnology, and mouse anti-FE65 (4H324) was from Abcam. FE65 is reported to be phosphorylated by several kinases at various residues, which can lead to reduction in electrophoretic mobility of FE65 (10, 33, 34). Therefore, multiple FE65 bands would be seen in Western blot analysis. Since the phosphorylation status of FE65 might vary from different samples, different banding patterns might be observed.

GST fusion protein binding assays

The GST-ARF6 and GST-FE65 PTB1 were expressed in *E. coli* BL21 and captured by glutathione-Sepharose 4B (GE Healthcare Biosciences) according to the manufacturer's

instructions. GST pulldown assays were performed essentially as described previously (32). In ARF6 pulldown assays, GST and GST-ARF6 baits were used to pull down FE65 from transfected cell lysates. Cells were harvested in ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; and Complete protease inhibitor; Roche). Following lysis, cells were cleared by centrifugation at 14,000 g at 4°C. The lysates were incubated with the baits at 4°C for 1 h. The captured proteins were boiled in SDS-PAGE sample buffer and then analyzed by SDS-PAGE and Western blotting.

For mammalian GST fusion protein binding assays, CHO cells were transfected with GST + ARF6, GST-FE65 WW + ARF6, GST-FE65 PTB1 + ARF6, or GST-FE65 PTB2 + ARF6. Cells were harvested in ice-cold lysis buffer as described above. Lysates were incubated with glutathione-Sepharose at 4°C for 1 h. The captured proteins were then analyzed by SDS-PAGE and immunoblotting. Similar assays were also performed for cells transfected with GST-FE65 PTB1 + ARF6, GST-FE65 PTB1 + ARF6 T27N, or GST-FE65 PTB1 + ARF6 Q67L. Mammalian GST-ARF6 fusion protein pulldown of FE65 were also performed by the method as described above. To perform direct protein-binding assay, His₆-ARF6 was expressed in *E. coli* BL21, purified by Ni-NTA agarose (Qiagen, Hilden, Germany), and incubated with purified GST or GST-FE65 PTB1 baits in ice-cold lysis buffer. The protein complexes were captured by glutathione-Sepharose 4B and analyzed by SDS-PAGE.

To determine the state of ARF6 (*i.e.*, ARF6-GDP or ARF6-GTP) that interacts with FE65, bacterial GST-FE65 PTB1 was used to pull down dominant negative ARF6 T27N and constitutively active ARF6 Q67L mutants from transfected CHO cell lysates. In brief, ARF6-transfected cells were harvested in ice-cold lysis/binding/wash buffer (25 mM Tris-HCl, pH 7.2; 150 mM NaCl; 5 mM MgCl₂; 1% Nonidet P-40; 5% glycerol; and protease inhibitor cocktail) and then cleared by centrifugation. The lysates were incubated with the bacterial GST-FE65 PTB1 baits at 4°C for 1 h. The captured proteins were boiled in SDS-PAGE sample buffer and then analyzed by SDS-PAGE and Western blotting.

To further confirm the state of ARF6 that interacts with FE65, wild-type ARF6-transfected cell lysates were loaded with either GDP or GTP by incubating with 1 mM GDP or 10 mM nonhydrolyzable GTPγS, respectively, at 30°C for 15 min and then followed by bacterial GST-FE65 PTB1 pulldown assays as described above.

Coimmunoprecipitation assays

CHO cells transfected with either FE65, FE65 + myc-tagged ARF6, or FE65 PTB1 + myc-tagged ARF6 were harvested in ice-cold lysis buffer. Myc-tagged ARF6 was immunoprecipitated from cell lysates by 9B11 anti-myc antibody at 4°C for 16 h. The antibody was captured by protein A-agarose (Sigma) at 4°C for 2 h. The immunoprecipitates were washed 3 times with ice-cold lysis buffer and then boiled in SDS/PAGE sample buffer for 10 min. Proteins in the immunoprecipitates were analyzed by SDS-PAGE and Western blotting. For endogenous interaction between FE65 and ARF6, the rat brain was homogenized in ice-cold lysis buffer and cleared by centrifugation as described above. ARF6 was immunoprecipitated from the lysate and was detected by 3A-1 anti-ARF6 antibody, whereas FE65 was detected by E20 anti-FE65 antibody.

ARF6 activation assays

ARF6 activation was determined using an active Arf6 pulldown kit (Cell Biolabs, San Diego, CA, USA). The principle of the kit is based on the fact that active ARF6 (*i.e.*, ARF6-GTP) binds specifically to the protein-binding domain (PBD) of GGA3 (33). To determine the ARF6-GTP level, cells were harvested in ice-cold lysis/binding/wash buffer and then cleared by centrifugation. The cleared lysates were incubated with GST-GGA3-PBD bait (*i.e.*, GST-GGA3-PBD fusion protein coupled on glutathione-Sepharose that is supplied with the kit) at 4°C for 3 h. The amounts of ARF6-GTP pulled down by the bait and total ARF6 in the lysates were analyzed by immunoblotting using 6ARF01 mouse monoclonal antibody.

Rac 1 activation assays

Rac1-GTP levels in cells were determined by using a Rac1 activation assay (ThermoScientific). In this assay, only Rac1-GTP interacts with GST-PAK1-PBD bait (34, 35). In brief, cells were harvested in assay/lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl; 1% Nonidet P-40; 10 mM MgCl₂; 1 mM EDTA; 2% glycerol; and protease inhibitor cocktail) and followed by centrifugation. The cleared lysates were incubated with GST-PAK1-PBD bait (*i.e.*, GST-PAK1-PBD fusion protein coupled on glutathione-Sepharose that is supplied with the kit) to pull down Rac1-GTP. The levels of Rac1-GTP pulled down by the bait and total Rac1 in the cell lysates were detected by Western blotting using 23A8 mouse monoclonal antibody.

Immunofluorescence studies and neurite length measurements

Neurons grown on coverslips were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS, blocked with 5% fetal bovine serum (FBS) in PBS for 30 min, and then probed with primary antibodies in 5% FBS/PBS. For FE65 and ARF6 colocalization studies, FE65 was detected using goat anti-FE65 (E20) and rat anti-ARF6. Primary antibodies were detected using AlexaFluor-coupled secondary Igs and nuclei labeled with 4',6-diamidino-2-phenylindole (DAPI) (all from Invitrogen). Coverslips were mounted in Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA). For intensity correlation analysis, images were captured using a Zeiss LSM510Meta confocal microscope equipped with a ×63, Plan-Apochromat 1.4 NA objective (Zeiss, Oberkochen, Germany), and analyzed using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) with the intensity correlation analysis plug-in essentially as described by us and others (29, 36, 37). Further calculations and statistical analyses were performed using Excel (Microsoft Corp., Redmond, WA, USA) and SPSS 15 (IBM, Chicago, IL, USA). Light-microscopy was performed using a Leica DM5000B microscope with ×63 HCX PL Fluotar phase objective.

For neurite length measurements, we employed a widely used approach that involves cotransfecting enhanced green fluorescent protein (EGFP)-expressing plasmid [pEGFP-C1 (Clontech) in this study] into neurons (31, 38, 39). Since GFP has a uniform distribution throughout neurons, it therefore was used to determine cell shape and also act as a marker for transfected neurons. In brief, pEGFP-C1 was cotransfected with different constructs and/or with different siRNAs into 2-day *in vitro* (DIV) primary rat cortical neurons. All GFP-expressing neurons were immunostained for cotransfected protein to confirm

expression. Healthy neurons were distinguished based on their morphologically normal nuclei stained by DAPI. The longest neurite of the transfected neurons was analyzed after 24 h for each treatment. Three independent experiments, with 40 neurons each, were performed in a blind manner. The lengths were determined as the distance from the growth cone tip to the periphery of the cell body and quantified using ImageJ with NeuronJ plug-in (40). Statistical analyses were performed using 1-way ANOVA test with Bonferroni *post hoc* test. Differences were considered significant at $P < 0.05$.

RESULTS

FE65 interacts directly with ARF6 through the PTB1 domain

From a yeast 2-hybrid screen of a human brain cDNA library using FE65 PTB1 + 2 domain as bait, we isolated a cDNA clone encoding the small GTPase ARF6. The FE65-ARF6 interaction in yeast was confirmed using LacZ liquid assays (data not shown).

To further analyze the FE65-ARF6 interaction, bacterially expressed GST and GST-ARF6 fusion proteins were used as baits to pull down FE65 from transfected cells. In this assay, GST-ARF6, but not GST, interacted with FE65 (Fig. 1A). We next tested the FE65-ARF6 interaction using immunoprecipitation assays from transfected cells. FE65 was transfected either alone or cotransfected with myc-tagged ARF6 and ARF6 then immunoprecipitated *via* the myc tag. FE65 was present in immunoprecipitates from the FE65 + ARF6 but not FE65 singly transfected control cells (Fig. 1B). To demonstrate that endogenous FE65 interacts with endogenous ARF6, we immunoprecipitated ARF6 from rat brain and probed these immunoprecipitates for FE65. FE65 was present in the ARF6 immunoprecipitates (Fig. 1C). Thus, FE65 and ARF6 interact in yeast 2-hybrid, GST pulldown, and immunoprecipitation assays, and endogenous FE65 interacts with endogenous ARF6 in immunoprecipitation assays from rat brain.

We next determined which domain of FE65 interacts with ARF6. To do so, we cotransfected CHO cells with full-length ARF6 and mammalian expression constructs encoding GST or GST fused to different domains of FE65. The domains were the WW domain, the first PTB domain (PTB1), and the second PTB domain (PTB2). GST pulldowns from the cell lysates were performed, and these were then probed on immunoblots for ARF6. These assays revealed that only GST-FE65 PTB1 interacted with ARF6 (Fig. 1D). Furthermore, FE65 PTB1 mutant could not interact with ARF6 in coimmunoprecipitation assay (Fig. 1E). To determine the region in ARF6 that mediates FE65-ARF6 interaction, we pulled down FE65 from transfected cell lysate using various GST-ARF6 deletion mutants. FE65 could be pulled down by GST-ARF6 28–175 (also ARF6 1–80 and full-length ARF6) but not ARF6 48–175 (Fig. 1F). This result suggests that aa 28–47 of ARF6 (a region that contains the guanine nucleotide binding switch I, residues 36–47, of ARF6) are critical for the interaction with FE65. To examine whether this interaction is direct (and not mediated by some other intermediary protein), we incubated *E. coli* purified His-ARF6 with *E. coli* purified GST or GST-FE65 PTB1 baits. Similar to the assays above, His-ARF6 was pulled down by GST-FE65 PTB1 but not GST (Fig. 1G). Taken together, these results demonstrate that ARF6 interacts directly with the FE65 PTB1 domain.

FE65 preferentially binds to the inactive ARF6-GDP form

Similar to other small GTPases, ARF6 exists in a GTP-bound active and a GDP-bound inactive state. These two states have distinct structures that alter the interactions between ARF6 and its binding proteins (for reviews, see refs. 21, 22). Therefore, it is possible that FE65 binds differentially to these two states of ARF6. To test this possibility, ARF6 Q67L and T27N mutants, which mimic the GTP- and GDP-bound states, respectively (for reviews, see refs. 21, 22), were utilized in both mammalian and bacterial GST-FE65 PTB1 pulldown assays (Fig. 2A, B). In the mammalian GST pulldown assay, GST-FE65 PTB1 was cotransfected with either wild-type ARF6 or the ARF6 mutants. GST-FE65 PTB1 was pulled down from the transfected cell lysates, and the amounts of bound ARF6 were detected by immunoblotting. FE65 PTB1 domain interacted more strongly with ARF6 T27N than wild-type ARF6 but did not interact with ARF6 Q67L (Fig. 2A). Likewise, similar pulldown assays using bacterial expressed GST-FE65 PTB1 as bait revealed that FE65 PTB1 domain bound strongly to ARF6 T27N but not ARF6 Q67L (Fig. 2B). The specificities of the ARF6 mutants were confirmed by GST-GGA3 pulldown assay in which the bait preferably binds to ARF6 Q67L mutant (Fig. 2B), which is similar to those published elsewhere (41, 42). To further preclude the possibility that this differential binding is an artifact of using the artificial mutants, we treated the wild-type ARF6 transfected cell lysates with either GDP or nonhydrolyzable GTP γ S to load either GDP or GTP nucleoside triphosphates onto ARF6 and then tested binding of GDP- and GTP-bound ARF6 to FE65 in pulldown assays. The GDP-bound and GTP-bound states of ARF6 were confirmed by performing ARF6 GTPase activation assays; GTP bound ARF6 interacts more strongly with GGA3 in these assays (Fig. 2C, bottom panel). In line with the assays involving mutants of ARF6, GDP-ARF6 interacted stronger than GTP-ARF6 with FE65 PTB1 domain (Fig. 2C, top panel). Thus, FE65 preferentially interacts with GDP- but not GTP-bound ARF6.

FE65 activates both endogenous ARF6 and Rac1

Typically, ARF effectors bind to ARF-GTP, and this suggests that FE65 does not function downstream of ARF6 as an effector protein. We therefore speculated that FE65 functions upstream to regulate ARF6 activation in some fashion. To test this possibility, we monitored the effect of modulating FE65 expression on the ARF6 activation. Overexpression of FE65 increased endogenous ARF6 activation (as detected using GGA3 pulldown ARF6 assays), whereas FE65 siRNA knockdown reduced ARF6 activation (Fig. 3A, B). These findings implicate that although it preferentially binds to ARF6-GDP form, FE65 promotes the activation of ARF6.

A large body of evidence indicates that the small GTPase Rac1 is a downstream effector of ARF6 activation (refs. 43–47; for review, see ref. 48). The above observations prompted us to investigate whether FE65 also activates endogenous Rac1. We therefore performed Rac1 activation assays (which involved assaying binding of Rac1 to its effector PAK1 in pulldown assays) in cells in which FE65 expression was modulated. Overexpression of FE65 by transfection increased Rac1 activation, whereas siRNA knockdown of FE65 reduced Rac1 activation in these assays (Figs. 3C, D). To determine whether this stimulatory effect of FE65 on Rac1 activation involved ARF6, we performed similar Rac1 activation assays in cells in which ARF6 levels were depleted using siRNAs. In control siRNA-

transfected cells, overexpression of FE65 again increased Rac1 activation but this stimulatory effect was abolished in ARF6 knockdown cells (Fig. 3E). Thus, FE65 acts upstream of ARF6 to regulate Rac1 activation.

FE65 and ARF6 colocalize in neuronal growth cones

Rac1 regulates actin dynamics within neurons, and during development, Rac1 controls neurite outgrowth *via* effects on the actin cytoskeleton within growth cones (for reviews, see refs. 49–51). Since FE65 regulates Rac1 activity, we therefore enquired whether FE65 and ARF6 colocalize in developing neurons and in particular, whether they colocalized in growth cones. Immunostaining for ARF6 revealed that it was principally a cytoplasmic protein in neurons that was enriched in perinuclear regions but also present in processes and growth cones (Fig. 4A, B). To determine whether FE65 and ARF6 colocalize in growth cones, we immunostained neurons and utilized intensity correlation analyses (ICAs; ref. 37) to determine whether there was significant overlap in the distribution of the two proteins in growth cones. ICA compares the scatter plots of 2 stains against the product of the difference of the pixel intensities of each of the two stains from their respective means. Thus, ICA determines whether the pixel intensities from 2 signals vary in synchrony and as such is superior to many other methods for determining the extent of colocalization of proteins in cells and tissues. The values obtained from the analyses can be reported as an intensity correlation quotient (ICQ), which is a statistically testable, single value assessment of the relationship between 2 stained protein pairs: for random staining, ICQ = 0; for dependent staining (colocalization), $0 < \text{ICQ} \leq +0.5$; and for segregated staining, $-0.5 \leq \text{ICQ} < 0$. Both ARF6 and FE65 were enriched in growth cones of developing rat cortical neurons (Fig. 4B), and ICA revealed that they colocalized to a highly significant level within this subcellular compartment (mean $\pm$ SEM ICQ=0.225 $\pm$ 0.01, $P<0.001$; $n=36$ cells).

FE65 and ARF6 stimulate neurite outgrowth

Since Rac1 regulates neurite outgrowth and we demonstrated that FE65 and ARF6 regulate Rac1 activity, we determined the effects of modulating FE65 and ARF6 expression on neurite outgrowth in rat cortical neurons. We first tested the effect of overexpressing FE65 and/or ARF6 on neurite outgrowth. To do so, we transfected DIV2 rat cortical neurons with either FE65; FE65 lacking PTB1 (FE65 Δ PTB1, which does not bind ARF6); or FE65 lacking PTB2 (FE65 Δ PTB2) together with ARF6 and monitored neurite outgrowth, as described previously (31). Expression of FE65 or ARF6 both stimulated neurite outgrowth, and this effect was more pronounced in FE65 + ARF6 cotransfected neurons. However, the effect of FE65 was lost in neurons expressing FE65 Δ PTB1 that does not bind ARF6 (Fig. 5A). Intriguingly, the stimulatory effect of FE65 was also markedly decreased when the PTB2 domain was deleted (Fig. 5A). This suggests that FE65 PTB2 domain also plays a role in mediating neurite extension.

We next tested how loss of ARF6 or FE65 influenced neurite outgrowth. Analyses of these neurons revealed that siRNA knockdown of either ARF6 or FE65 both reduced neurite outgrowth (Fig. 5B, C). However, overexpression of FE65 did not rescue the effect of ARF6 knockdown (Fig. 5B) and FE65 knockdown did not influence the stimulatory effect of ARF6 on neurite outgrowth (Fig. 5C). Immunoblots revealed that both the FE65 and ARF6

siRNAs induced efficient knockdown in the neurons (Fig. 5D). These results support the notion that FE65 acts upstream of ARF6 to promote neurite outgrowth and are thus complementary to the biochemical studies (Figs. 2 and 3), which place FE65 as an upstream regulator of ARF6 and Rac1.

Rac1 is indispensable for FE65 and ARF6 stimulation of neurite outgrowth

As shown in the biochemical assays, FE65 stimulates ARF6-Rac1 signaling (Fig. 3C, D). It is therefore possible that the effect of FE65 on neurite outgrowth involves Rac1. To test this possibility, we monitored the stimulatory effect of FE65 + ARF6 expression on neurite outgrowth in cells expressing a dominant-negative Rac1 (N17Rac1; ref. 31). Overexpression of wild-type Rac1 enhanced neurite outgrowth in FE65 + ARF6 cotransfected neurons. However, N17Rac1 blocked this effect of FE65 + ARF6 (Fig. 6A). In agreement with this, the stimulatory effect of FE65 and ARF6 on neurite extension was markedly reduced in Rac1-knockdown neurons (Fig. 6B). On the other hand, expression of constitutively active Rac1 (V12Rac1) alone was sufficient to stimulate neurite outgrowth (Fig. 6A, C). Knockdown of either FE65 or ARF6 induced small decrease in neurite extension of the V12Rac1 transfected neurons (Fig. 6C). Such a reduction in neurite outgrowth might due to the inactivation of endogenous Rac1 in the FE65 and ARF6 knockdown cells. Nevertheless, Rac1 appears to be essential for the effect of FE65 and ARF6 on neurite outgrowth.

To test if the effect of Rac1 on neurite outgrowth is *via* actin-modeling, rat cortical neurons were treated with actin-destabilizing agent CytoD. Decrease of neurite outgrowth was observed in the empty vector (EV)-transfected neurons treated with CytoD (Fig. 6D). The stimulatory effect of V12Rac1 on neurite extension was suppressed in the presence of CytoD (Fig. 6D). This suggests that Rac1 stimulates neurite outgrowth in rat cortical neurons, at least in part, *via* actin modeling.

DISCUSSION

In this study, we identify the small GTPase ARF6 as a binding partner for the neuronal adaptor protein FE65. FE65 contains a number of protein-protein interaction domains, including an N-terminal WW domain and 2 C-terminal PTB domains, and we show that binding of ARF6 is mediated *via* the first (N-terminal) PTB1 domain. FE65 also binds to 2 nuclear proteins, the histone acetyl transferase Tip60, and the transcription factor CP2/LSF/LBP1 *via* PTB1 (9, 52, 53). Since ARF6 is a cytoplasmic protein (Fig. 4A), it is unlikely to compete CP2/LSF/LBP1 for binding to FE65. However, it is not known whether Tip60 competes with ARF6 for FE65 PTB1, as a splice variant of Tip60, PLA2 interacting protein, localizes to both cytoplasm and nucleus (54). In addition, several low-density lipoprotein receptor family members, including LRP1, ApoER2, and VLDLR, have been shown to interact with FE65 PTB1 (55–60). Of note, LRP1 has been recently shown to act as a myelin-associated glycoprotein receptor to inhibit neurite outgrowth (61), ApoER2 functions as a Reelin receptor to regulate neurite motility (62), and VLDLR has also been implicated in neurite development (63). Since we show here that binding of ARF6 to FE65 PTB1 promotes neurite outgrowth, it is possible that changes in binding of these different FE65 PTB1 ligands is a mechanism for regulating and fine-tuning neurite outgrowth in

different neuronal populations during development. Indeed, such mechanisms may also be conserved in the adult to regulate plasticity.

The mechanisms for binding of PTB domains with their ligands has now been characterized in a number of studies (for review, see ref. 64). Many PTB domain ligands interact *via* canonical C-terminal NPXY or NXXY sequences in which the tyrosine can be either phosphorylated or nonphosphorylated (64). Although ARF6 does not contain such sequences, the PTB domains are now known to interact with ligands that do not contain NPXY/NXXY sequences. For example, binding of Deleted in liver cancer-1 to the tensin2 PTB domain has recently been shown to involve a novel interface and not the classical NPXY/NXXY sequence (65). Association of ARF6 with the FE65 PTB1 domain thus appears to resemble this less well-characterized mode of interaction.

Since FE65 interacts with the guanine nucleotide binding switch I of ARF6, this may provide an explanation for the fact that FE65 preferably binds to the ARF6-GDP as switch I undergoes conformational reorganization during GDP/GTP cycle of ARF6 (66, 67). Notably, such FE65-ARF6-GDP interaction stimulates ARF6 activation. The mechanism that underlies this stimulatory effect is unclear; certainly FE65 does not structurally resemble any known GEF. Of note, several ARF-interacting adaptor proteins that lack of intrinsic GEF and GAP activities have been shown to alter ARF activity. For example, Arfaptin1 was found to inhibit ARF even though it binds to constitutively active mutant of ARF (68, 69). As an adaptor protein, it seems likely that FE65 may somehow mediate the recruitment of other proteins (perhaps ARF6 GEFs) to modulate ARF6 activity. Indeed, there are precedents for such a model. For example, GULP1 binds to ARF6 and the ARF6-GAP ACAP1 to stimulate ARF6 activation (70).

ARF6 regulates a number of physiological processes, including endocytosis, secretion, phagocytosis, cell adhesion, and cell migration; in neurons, ARF6 also regulates axon and neurite outgrowth (refs. 21, 22, 71–73; and results described here). This role in neurite outgrowth is mediated, at least in part, *via* Rac1 dependent actin remodeling in the growth cone; ARF6 localizes to the plasma membrane and recruits Rac1 to the cell surface (for reviews, see refs. 21, 22). Interestingly, APP is also present within growth cones and functions in axonal outgrowth (19, 74–76), but the mechanisms that underlie this function are not properly understood. Both APP and FE65 have been implicated in various cytoplasmic and nuclear processes (14). Of note, APP has been shown to function as a cytosolic docking site to retain FE65 in the cytoplasm through the interaction between FE65 PTB2 and APP intracellular domain (77). Therefore, it is possible that the role of APP in axonal outgrowth may be to recruit FE65 to growth cone membranes where it can then interact with ARF6 *via* PTB1. In support of this notion, the stimulatory effect of FE65 on neurite outgrowth was significantly reduced when the PTB2 domain was deleted (*i.e.*, FE65 PTB2). Since FE65 PTB2 remains concentrated at the growth cone (Supplemental Fig. S1), such recruitment of FE65 by APP may occur after FE65 entry to growth cones. Once recruited, the FE65-ARF6 interaction might then stimulate the activation of ARF6 and downstream Rac1 so as to modulate actin dynamics. Interestingly, FE65 also binds *via* its WW domain to mammalian enabled (Mena), a member of the Ena/Vasp family of actin regulatory proteins (78). Furthermore, FE65 and Rac1 have been shown to interact in a

coimmunoprecipitation assay (79). Thus, FE65 may perform critical functions within growth cones to recruit various molecules and integrate a variety of signaling cascades that control axon outgrowth.

In our study, actin-destabilizing agent CytoD abolished the stimulatory effect of V12Rac1 on neurite extension (Fig. 6). It may suggest that destabilization of actin has an inhibitory effect on the process. However, the role of actin depolymerization in neurite development remains controversial (80–90). The causes for such contradictory data are not fully understood; however, they may due to the differences of neuron types, ages, animal strains, culture conditions, and experimental approaches employed. Further studies are required to find out the reasons for such conflicting observations.

Many of the cellular mechanisms that regulate axon and dendritic outgrowth during development are conserved in the adult and function at the synapse to control plasticity. As such, the FE65-ARF6-Rac1 interaction we describe here may also function in synapses. Synaptic dysfunction and loss are key features of Alzheimer's disease. Disruption to ARF6 function has recently been implicated in the pathogenesis of Alzheimer's disease *via* ARF6 control of BACE1 trafficking; BACE1 processing of APP is required for production of A β (91). Our findings suggest that perturbation of FE65/ARF6 function might also disrupt synaptic function *via* alterations in Rac1 dependent actin remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funds from the Research Grant Council Hong Kong (CUHK467712); the CUHK Direct Grant Scheme (2030443); United College Endowment Fund (CA11156 and CA11188); Wellcome Trust; Medical Research Council (UK); and Alzheimer's Research UK. The authors thank Ka Ming Vincent Tam for technical assistance.

Abbreviations

Aβ	amyloid- β
APP	amyloid precursor protein
ARF6	ADP-ribosylation factor 6
CHO	Chinese hamster ovary
DAPI	4',6-diamidino-2-phenylindole
DIV	days <i>in vitro</i>
EGFP	enhanced green fluorescent protein
EV	empty vector
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor

GFP	green fluorescent protein
GST	glutathione <i>S</i> transferase
ICA	intensity correlation analysis
ICQ	intensity correlation quotient
PBD	protein-binding domain
PTB	phosphotyrosine binding

REFERENCES

1. Borg JP, Ooi J, Levy E, Margolis B. The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol. Cell. Biol.* 1996; 16:6229–6241. [PubMed: 8887653]
2. Fiore F, Zambrano N, Minopoli G, Donini V, Duilio A, Russo T. The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. *J. Biol. Chem.* 1995; 270:30853–30856. [PubMed: 8537337]
3. McLoughlin DM, Miller CC. The intracellular cytoplasmic domain of the Alzheimer's disease amyloid precursor protein interacts with phosphotyrosine-binding domain proteins in the yeast two-hybrid system. *FEBS Lett.* 1996; 397:197–200. [PubMed: 8955346]
4. Ando K, Iijima KI, Elliott JI, Kirino Y, Suzuki T. Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of b-amyloid. *J. Biol. Chem.* 2001; 276:40353–40361. [PubMed: 11517218]
5. Guenette S, Chang Y, Hiesberger T, Richardson JA, Eckman CB, Eckman EA, Hammer RE, Herz J. Essential roles for the FE65 amyloid precursor protein-interacting proteins in brain development. *EMBO J.* 2006; 25:420–431. [PubMed: 16407979]
6. King GD, Scott Turner R. Adaptor protein interactions: modulators of amyloid precursor protein metabolism and Alzheimer's disease risk? *Exp. Neurol.* 2004; 185:208–219. [PubMed: 14736502]
7. Sabo SL, Lanier LM, Ikin AF, Khorkova O, Sahasrabudhe S, Greengard P, Buxbaum JD. Regulation of b-amyloid secretion by FE65, an amyloid protein precursor-binding protein. *J. Biol. Chem.* 1999; 274:7952–7957. [PubMed: 10075692]
8. Wang B, Hu Q, Hearn MG, Shimizu K, Ware CB, Liggitt DH, Jin LW, Cool BH, Storm DR, Martin GM. Isoform-specific knockout of FE65 leads to impaired learning and memory. *J. Neurosci. Res.* 2004; 75:12–24. [PubMed: 14689444]
9. Cao X, Sudhof TC. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science.* 2001; 293:115–120. [PubMed: 11441186]
10. Perkinton MS, Standen CL, Lau KF, Kesavapany S, Byers HL, Ward M, McLoughlin DM, Miller CC. The c-Abl tyrosine kinase phosphorylates the Fe65 adaptor protein to stimulate Fe65/amyloid precursor protein nuclear signaling. *J. Biol. Chem.* 2004; 279:22084–22091. [PubMed: 15031292]
11. Nakaya T, Kawai T, Suzuki T. Regulation of FE65 nuclear translocation and function by amyloid beta-protein precursor in osmotically stressed cells. *J. Biol. Chem.* 2008; 283:19119–19131. [PubMed: 18468999]
12. Stante M, Minopoli G, Passaro F, Raia M, Vecchio LD, Russo T. Fe65 is required for Tip60-directed histone H4 acetylation at DNA strand breaks. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:5093–5098. [PubMed: 19282473]
13. McLoughlin DM, Irving NG, Miller CC. The Fe65 and X11 families of proteins: proteins that interact with the Alzheimer's disease amyloid precursor protein. *Biochem. Soc. Trans.* 1998; 26:497–500. [PubMed: 9765903]
14. Minopoli G, Gargiulo A, Parisi S, Russo T. Fe65 matters: New light on an old molecule. *IUBMB Life.* 2012; 64:936–942. [PubMed: 23129269]

15. Turner AJ, Belyaev ND, Nalivaeva NN. Mediator: the missing link in amyloid precursor protein nuclear signalling. *EMBO Rep.* 2011; 12:180–181. [PubMed: 21368841]
16. Kesavapany S, Banner SJ, Lau KF, Shaw CE, Miller CC, Cooper JD, McLoughlin DM. Expression of the Fe65 adapter protein in adult and developing mouse brain. *Neuroscience.* 2002; 115:951–960. [PubMed: 12435432]
17. Ma QH, Futagawa T, Yang WL, Jiang XD, Zeng L, Takeda Y, Xu RX, Bagnard D, Schachner M, Furley AJ, Karagogeos D, Watanabe K, Dawe GS, Xiao ZC. A TAG1-APP signalling pathway through Fe65 negatively modulates neurogenesis. *Nat. Cell Biol.* 2008; 10:283–294. [PubMed: 18278038]
18. Sabo SL, Ikin AF, Buxbaum JD, Greengard P. The Alzheimer amyloid precursor protein (APP) and FE65, an APP-binding protein, regulate cell movement. *J. Cell Biol.* 2001; 153:1403–1414. [PubMed: 11425871]
19. Sabo SL, Ikin AF, Buxbaum JD, Greengard P. The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses in vitro and in vivo. *J. Neurosci.* 2003; 23:5407–5415. [PubMed: 12843239]
20. Wang Y, Zhang M, Moon C, Hu Q, Wang B, Martin G, Sun Z, Wang H. The APP-interacting protein FE65 is required for hippocampus-dependent learning and long-term potentiation. *Learn. Mem.* 2009; 16:537–544. [PubMed: 19713352]
21. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell. Biol.* 2006; 7:347–358. [PubMed: 16633337]
22. Jaworski J. ARF6 in the nervous system. *Eur. J. Cell Biol.* 2007; 86:513–524. [PubMed: 17559968]
23. Hernandez-Deviez DJ, Roth MG, Casanova JE, Wilson JM. ARNO and ARF6 regulate axonal elongation and branching through downstream activation of phosphatidylinositol 4-phosphate 5-kinase alpha. *Mol. Biol. Cell.* 2004; 15:111–120. [PubMed: 14565977]
24. Eva R, Crisp S, Marland JR, Norman JC, Kanamarlapudi V, Ffrench-Constant C, Fawcett JW. ARF6 directs axon transport and traffic of integrins and regulates axon growth in adult DRG neurons. *J. Neurosci.* 2012; 32:10352–10364. [PubMed: 22836268]
25. Schweitzer JK, Sedgwick AE, D'Souza-Schorey C. ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis. *Semin. Cell Dev. Biol.* 2011; 22:39–47. [PubMed: 20837153]
26. De Curtis I. Functions of Rac GTPases during neuronal development. *Dev. Neurosci.* 2008; 30:47–58. [PubMed: 18075254]
27. Hao CY, Perkinton MS, Chan WW, Chan HY, Miller CC, Lau KF. GULP1 is a novel APP-interacting protein that alters APP processing. *Biochem. J.* 2011; 436:631–639. [PubMed: 21486224]
28. Chow WN, Luk HW, Chan HY, Lau KF. Degradation of mutant huntingtin via the ubiquitin/proteasome system is modulated by FE65. *Biochem. J.* 2012; 443:681–689. [PubMed: 22352297]
29. Vagnoni A, Perkinton MS, Gray EH, Francis PT, Noble W, Miller CC. Calsyntenin-1 mediates axonal transport of the amyloid precursor protein and regulates Abeta production. *Hum. Mol. Genet.* 2012; 21:2845–2854. [PubMed: 22434822]
30. Lau KF, McLoughlin DM, Standen CL, Irving NG, Miller CC. Fe65 and X11b co-localize with and compete for binding to the amyloid precursor protein. *Neuroreport.* 2000; 11:3607–3610. [PubMed: 11095528]
31. Tudor EL, Perkinton MS, Schmidt A, Ackerley S, Brownlees J, Jacobsen NJ, Byers HL, Ward M, Hall A, Leigh PN, Shaw CE, McLoughlin DM, Miller CC. ALS2/Alsin regulates Rac-PAK signaling and neurite outgrowth. *J. Biol. Chem.* 2005; 280:34735–34740. [PubMed: 16049005]
32. Lau KF, Chan WM, Perkinton MS, Tudor EL, Chang RC, Chan HY, McLoughlin DM, Miller CC. Dexas1 interacts with FE65 to regulate FE65-amyloid precursor protein-dependent transcription. *J. Biol. Chem.* 2008; 283:34728–34737. [PubMed: 18922798]
33. Santy LC, Casanova JE. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* 2001; 154:599–610. [PubMed: 11481345]

34. Taylor SJ, Shalloway D. Cell cycle-dependent activation of Ras. *Curr. Biol.* 1996; 6:1621–1627. [PubMed: 8994826]
35. Benard V, Bohl BP, Bokoch GM. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* 1999; 274:13198–13204. [PubMed: 10224076]
36. Tudor EL, Galtrey CM, Perkinton MS, Lau KF, De Vos KJ, Mitchell JC, Ackerley S, Hortobagyi T, Vamos E, Leigh PN, Klasen C, McLoughlin DM, Shaw CE, Miller CC. Amyotrophic lateral sclerosis mutant vesicle-associated membrane protein-associated protein-B transgenic mice develop TAR-DNA-binding protein-43 pathology. *Neuroscience.* 2010; 167:774–785. [PubMed: 20188146]
37. Li Q, Lau A, Morris TJ, Guo L, Fordyce CB, Stanley EF. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J. Neurosci.* 2004; 24:4070–4081. [PubMed: 15102922]
38. Bryan B, Kumar V, Stafford LJ, Cai Y, Wu G, Liu M. GEFT, a Rho family guanine nucleotide exchange factor, regulates neurite outgrowth and dendritic spine formation. *J. Biol. Chem.* 2004; 279:45824–45832. [PubMed: 15322108]
39. May V, Schiller MR, Eipper BA, Mains RE. Kalirin Dbl-homology guanine nucleotide exchange factor 1 domain initiates new axon outgrowths via RhoG-mediated mechanisms. *J. Neurosci.* 2002; 22:6980–6990. [PubMed: 12177196]
40. Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry A.* 2004; 58:167–176. [PubMed: 15057970]
41. Macia E, Luton F, Partisani M, Cherfils J, Chardin P, Franco M. The GDP-bound form of Arf6 is located at the plasma membrane. *J. Cell Sci.* 2004; 117:2389–2398. [PubMed: 15126638]
42. Nekhoroshkova E, Albert S, Becker M, Rapp UR. A-RAF kinase functions in ARF6 regulated endocytic membrane traffic. *PLoS One.* 2009; 4:e4647. [PubMed: 19247477]
43. Boshans RL, Szanto S, van Aelst L, D'Souza-Schorey C. ADP-ribosylation factor 6 regulates actin cytoskeleton remodeling in coordination with Rac1 and RhoA. *Mol. Cell. Biol.* 2000; 20:3685–3694. [PubMed: 10779358]
44. Radhakrishna H, Al-Awar O, Khachikian Z, Donaldson JG. ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J. Cell Sci.* 1999; 112:855–866. [PubMed: 10036235]
45. Balasubramanian N, Scott DW, Castle JD, Casanova JE, Schwartz MA. Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. *Nat. Cell Biol.* 2007; 9:1381–1391. [PubMed: 18026091]
46. Monteleon CL, Sedgwick A, Hartsell A, Dai M, Whittington C, Voytik-Harbin S, D'Souza-Schorey C. Establishing epithelial glandular polarity: interlinked roles for ARF6, Rac1, and the matrix microenvironment. *Mol. Biol. Cell.* 2012; 23:4495–4505. [PubMed: 23051733]
47. Chen PW, Jian X, Yoon HY, Randazzo PA. ARAP2 signals through Arf6 and Rac1 to control focal adhesion morphology. *J. Biol. Chem.* 2013; 288:5849–5860. [PubMed: 23295182]
48. Myers KR, Casanova JE. Regulation of actin cytoskeleton dynamics by Arf-family GTPases. *Trends Cell Biol.* 2008; 18:184–192. [PubMed: 18328709]
49. Nikolic M. The role of Rho GTPases and associated kinases in regulating neurite outgrowth. *Int. J. Biochem. Cell Biol.* 2002; 34:731–745. [PubMed: 11950591]
50. Lundquist EA. Rac proteins and the control of axon development. *Curr. Opin. Neurobiol.* 2003; 13:384–390. [PubMed: 12850224]
51. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature.* 2002; 420:629–635. [PubMed: 12478284]
52. Kim HS, Kim EM, Lee JP, Park CH, Kim S, Seo JH, Chang KA, Yu E, Jeong SJ, Chong YH, Suh YH. C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3 β expression. *FASEB J.* 2003; 17:1951–1953. [PubMed: 12923068]
53. Zambrano N, Minopoli G, de Candia P, Russo T. The Fe65 adaptor protein interacts through its PID1 domain with the transcription factor CP2/LSF/LBP1. *J. Biol. Chem.* 1998; 273:20128–20133. [PubMed: 9685356]

54. Sheridan AM, Force T, Yoon HJ, O'Leary E, Choukroun G, Taheri MR, Bonventre JV. PLIP, a novel splice variant of Tip60, interacts with group IV cytosolic phospholipase A(2), induces apoptosis, and potentiates prostaglandin production. *Mol. Cell. Biol.* 2001; 21:4470–4481. [PubMed: 11416127]
55. Dumanis SB, Chamberlain KA, Jin Sohn Y, Jin Lee Y, Guenette SY, Suzuki T, Mathews PM, Pak D, Rebeck GW, Suh YH, Park HS, Hoe HS. FE65 as a link between VLDLR and APP to regulate their trafficking and processing. *Mol. Neurodegener.* 2012; 7:9. [PubMed: 22429478]
56. Hoe HS, Magill LA, Guenette S, Fu Z, Vicini S, Rebeck GW. FE65 interaction with the ApoE receptor ApoEr2. *J. Biol. Chem.* 2006; 281:24521–24530. [PubMed: 16638748]
57. Trommsdorff M, Borg JP, Margolis B, Herz J. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J. Biol. Chem.* 1998; 273:33556–33560. [PubMed: 9837937]
58. Pietrzik CU, Yoon IS, Jaeger S, Busse T, Weggen S, Koo EH. FE65 constitutes the functional link between the low-density lipoprotein receptor-related protein and the amyloid precursor protein. *J. Neurosci.* 2004; 24:4259–4265. [PubMed: 15115822]
59. Mulvihill MM, Guttman M, Komives EA. Protein interactions between Fe65, the LDL receptor-related protein and the amyloid precursor protein. *Biochemistry.* 2011; 50:6208–6216. [PubMed: 21650223]
60. Kounnas MZ, Moir RD, Rebeck GW, Bush AI, Argraves WS, Tanzi RE, Hyman BT, Strickland DK. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell.* 1995; 82:331–340. [PubMed: 7543026]
61. Stiles TL, Dickendesher TL, Gaultier A, Fernandez-Castaneda A, Mantuano E, Giger RJ, Gonias SL. LDL receptor-related protein-1 is a sialic-acid-independent receptor for myelin-associated glycoprotein that functions in neurite outgrowth inhibition by MAG and CNS myelin. *J. Cell Sci.* 2013; 126:209–220. [PubMed: 23132925]
62. Leemhuis J, Bouche E, Frotscher M, Henle F, Hein L, Herz J, Meyer DK, Pichler M, Roth G, Schwan C, Bock HH. Reelin signals through apolipoprotein E receptor 2 and Cdc42 to increase growth cone motility and filopodia formation. *J. Neurosci.* 2010; 30:14759–14772. [PubMed: 21048135]
63. Sepp KJ, Hong P, Lizarraga SB, Liu JS, Mejia LA, Walsh CA, Perrimon N. Identification of neural outgrowth genes using genome-wide RNAi. *PLoS Genet.* 2008; 4:e1000111. [PubMed: 18604272]
64. Uhlik MT, Temple B, Bencharit S, Kimple AJ, Siderovski DP, Johnson GL. Structural and evolutionary division of phosphotyrosine binding (PTB) domains. *J. Mol. Biol.* 2005; 345:1–20. [PubMed: 15567406]
65. Chen L, Liu C, Ko FC, Xu N, Ng IO, Yam JW, Zhu G. Solution structure of the phosphotyrosine binding (PTB) domain of human tensin2 protein in complex with deleted in liver cancer 1 (DLC1) peptide reveals a novel peptide binding mode. *J. Biol. Chem.* 2012; 287:26104–26114. [PubMed: 22645138]
66. Pasqualato S, Menetrey J, Franco M, Cherfils J. The structural GDP/GTP cycle of human Arf6. *EMBO Rep.* 2001; 2:234–238. [PubMed: 11266366]
67. Menetrey J, Macia E, Pasqualato S, Franco M, Cherfils J. Structure of Arf6-GDP suggests a basis for guanine nucleotide exchange factors specificity. *Nat. Struct. Biol.* 2000; 7:466–469. [PubMed: 10881192]
68. Kanoh H, Williger BT, Exton JH. Arfapatin 1, a putative cytosolic target protein of ADP-ribosylation factor, is recruited to Golgi membranes. *J. Biol. Chem.* 1997; 272:5421–5429. [PubMed: 9038142]
69. Tsai SC, Adamik R, Hong JX, Moss J, Vaughan M, Kanoh H, Exton JH. Effects of arfapatin 1 on guanine nucleotide-dependent activation of phospholipase D and cholera toxin by ADP-ribosylation factor. *J. Biol. Chem.* 1998; 273:20697–20701. [PubMed: 9694811]
70. Ma Z, Nie Z, Luo R, Casanova JE, Ravichandran KS. Regulation of Arf6 and ACAP1 signaling by the PTB-domain-containing adaptor protein GULP. *Curr. Biol.* 2007; 17:722–727. [PubMed: 17398097]

71. Albertinazzi C, Za L, Paris S, de Curtis I. ADP-ribosylation factor 6 and a functional PIX/p95-APP1 complex are required for Rac1B-mediated neurite outgrowth. *Mol. Biol. Cell.* 2003; 14:1295–1307. [PubMed: 12686588]
72. Mo J, Choi S, Ahn PG, Sun W, Lee HW, Kim H. PDZ-scaffold protein, Tamalin promotes dendritic outgrowth and arborization in rat hippocampal neuron. *Biochem. Biophys. Res. Commun.* 2012; 422:250–255. [PubMed: 22569042]
73. Yamazaki T, Koo EH, Selkoe DJ. Cell surface amyloid beta-protein precursor colocalizes with beta 1 integrins at substrate contact sites in neural cells. *J. Neurosci.* 1997; 17:1004–1010. [PubMed: 8994055]
74. Hoe HS, Lee KJ, Carney RS, Lee J, Markova A, Lee JY, Howell BW, Hyman BT, Pak DT, Bu G, Rebeck GW. Interaction of reelin with amyloid precursor protein promotes neurite outgrowth. *J. Neurosci.* 2009; 29:7459–7473. [PubMed: 19515914]
75. Perez RG, Zheng H, Van der Ploeg LH, Koo EH. The beta-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. *J. Neurosci.* 1997; 17:9407–9414. [PubMed: 9390996]
76. Rama N, Goldschneider D, Corset V, Lambert J, Pays L, Mehlen P. Amyloid precursor protein regulates netrin-1-mediated commissural axon outgrowth. *J. Biol. Chem.* 2012; 287:30014–30023. [PubMed: 22782894]
77. Minopoli G, de Candia P, Bonetti A, Faraonio R, Zambrano N, Russo T. The b-amyloid precursor protein functions as a cytosolic anchoring site that prevents Fe65 nuclear translocation. *J. Biol. Chem.* 2001; 276:6545–6550. [PubMed: 11085987]
78. Ermeikova KS, Zambrano N, Linn H, Minopoli G, Gertler F, Russo T, Sudol M. The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of *Drosophila* enabled. *J. Biol. Chem.* 1997; 272:32869–32877. [PubMed: 9407065]
79. Wang PL, Niidome T, Kume T, Akaike A, Kihara T, Sugimoto H. Functional and molecular interactions between Rac1 and FE65. *Neuroreport.* 2011; 22:716–720. [PubMed: 21878794]
80. Yamada KM, Spooner BS, Wessells NK. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 1970; 66:1206–1212. [PubMed: 5273449]
81. Bentley D, Toroian-Raymond A. Disoriented path-finding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature.* 1986; 323:712–715. [PubMed: 3773996]
82. Marsh L, Letourneau PC. Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. *J. Cell Biol.* 1984; 99:2041–2047. [PubMed: 6389568]
83. Luo L. Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu. Rev. Cell Dev. Biol.* 2002; 18:601–635. [PubMed: 12142283]
84. Flynn KC, Hellal F, Neukirchen D, Jacob S, Tahirovic S, Dupraz S, Stern S, Garvalov BK, Gurniak C, Shaw AE, Meyn L, Wedlich-Soldner R, Bamburg JR, Small JV, Witke W, Bradke F. ADF/cofilin-mediated actin retrograde flow directs neurite formation in the developing brain. *Neuron.* 2012; 76:1091–1107. [PubMed: 23259946]
85. Lu W, Fox P, Lakonishok M, Davidson MW, Gelfand VI. Initial neurite outgrowth in *Drosophila* neurons is driven by kinesin-powered microtubule sliding. *Curr. Biol.* 2013; 23:1018–1023. [PubMed: 23707427]
86. Dent EW, Kwiatkowski AV, Mebane LM, Philippar U, Barzik M, Robinson DA, Gupton S, Van Veen JE, Furman C, Zhang J, Alberts AS, Mori S, Gertler FB. Filopodia are required for cortical neurite initiation. *Nat. Cell Biol.* 2007; 9:1347–1359. [PubMed: 18026093]
87. Williams KL, Rahimtula M, Mearow KM. Hsp27 and axonal growth in adult sensory neurons in vitro. *BMC Neurosci.* 2005; 6:24. [PubMed: 15819993]
88. Frey D, Laux T, Xu L, Schneider C, Caroni P. Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *J. Cell Biol.* 2000; 149:1443–1454. [PubMed: 10871284]
89. Montenegro-Venegas C, Tortosa E, Rosso S, Peretti D, Bollati F, Bisbal M, Jausoro I, Avila J, Caceres A, Gonzalez-Billault C. MAP1B regulates axonal development by modulating Rho-GTPase Rac1 activity. *Mol. Biol. Cell.* 2010; 21:3518–3528. [PubMed: 20719958]

90. Tahirovic S, Hellal F, Neukirchen D, Hindges R, Garvalov BK, Flynn KC, Stradal TE, Chrostek-Grashoff A, Brakebusch C, Bradke F. Rac1 regulates neuronal polarization through the WAVE complex. *J. Neurosci.* 2010; 30:6930–6943. [PubMed: 20484635]
91. Sannerud R, Declerck I, Peric A, Raemaekers T, Menendez G, Zhou L, Veerle B, Coen K, Munk S, De Strooper B, Schiavo G, Annaert W. ADP ribosylation factor 6 (ARF6) controls amyloid precursor protein (APP) processing by mediating the endosomal sorting of BACE1. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:E559–568. [PubMed: 21825135]

Figure 1.

FE65 interacts with ARF6 via its PTB1 domain. A) ARF6 binds to FE65 in GST pull-down assays. *E. coli*-expressed GST and GST-ARF6 were used as baits in pull-down assays from FE65-transfected cells. FE65 in the cell lysates and pull-downs were detected using a goat anti-FE65. Coomassie blue gel showed the GST and GST-ARF6 baits. B) FE65 and ARF6 interact in immunoprecipitation assays from transfected cells. Immunoprecipitations were performed from CHO cells transfected with FE65 or FE65 + myc-tagged ARF6. ARF6 was immunoprecipitated using anti-myc antibody 9B11. Immunoprecipitated FE65 and ARF6

were detected by a goat anti-FE65 and a rat anti-ARF6, respectively. Symbols – and + indicate the absence or presence of myc antibody 9B11 in the immunoprecipitations. *C*) FE65 interacts endogenously with ARF6. ARF6 was immunoprecipitated from rat brain lysate using anti-ARF6 (3A-1). ARF6 was detected using rat anti-ARF6 antibody and FE65 using goat anti FE65 antibody. Immunoprecipitated FE65 and ARF6 were detected by a goat anti-FE65 and a rat anti-ARF6, respectively. Symbols – and + indicate the absence or presence of anti-ARF6 (3A-1) in the immunoprecipitations. *D*) ARF6 interacts with FE65 *via* FE65 PTB1 domain. Myc-tagged ARF6 was cotransfected with GST, GST-FE65 WW, PTB1, or PTB2 domains into CHO cells. GST-FE65 fusion proteins were then pulled down, and the samples were probed on immunoblots for ARF6 and the GST baits as indicated. Approximately 12% of ARF6 in the input lysate was pulled down by GST-FE65 PTB1. *E*) FE65 PTB1 domain is essential for the interaction. Myc-tagged ARF6 was cotransfected with FE65 or FE65 PTB1 into CHO cells. ARF6 was immunoprecipitated from the lysates using myc antibody 9B11. ARF6 and FE65 in the precipitates were detected using rat anti-ARF6 antibody and FE65 using goat anti FE65 antibody, respectively. Symbols – and + indicate the absence or presence of myc antibody 9B11 in the immunoprecipitations. *F*) Residues 28 to 47 of ARF6 is important for FE65-ARF6 interaction. Myc-tagged FE65 was cotransfected with GST, GST-ARF6, ARF6 1–80, ARF6 28–175, ARF6 48–175, or ARF6 73–175 into CHO cells. GST-ARF6 fusion proteins were then pulled down and the samples probed on immunoblots for FE65 and the GST baits as indicated. Schematic diagram shows the ARF6 mutants. *G*) FE65 PTB1 domain interacts directly with ARF6. *E. coli*-expressed GST and GST-FE65 PTB1 were used to pull down purified His-tagged ARF6. Left panel: Coomassie-stained gel of the recombinant proteins. Right panel: pulldown assays.

Figure 2.

FE65 preferentially binds to GDP bound ARF6. **A)** Mammalian expression construct of GST-FE65 PTB1 was cotransfected with ARF6, ARF6 T27N (mimicking ARF6-GDP), or ARF6 Q67L (mimicking ARF6-GTP). FE65 was pulled down using the GST tag, and the amounts of bound ARF6 were detected by immunoblotting. Samples of both the input lysates and pulldowns are shown. GST-FE65 PTB1 bait pulled down 10, 23, and 2% of ARF6, ARF6 T27N, and ARF6 Q67L from the corresponding input lysates, respectively. **B)** *E. coli*-expressed GST-FE65 PTB1 domain was used as bait in pulldown assays from CHO cells transfected with myc-tagged ARF6, ARF6 T27N, or ARF6 Q67L. ARF6 in the input lysates and pulldowns was detected by anti-myc 9B11. Coomassie blue gel (third panel) showed GST-FE65 PTB1 baits used in the pulldowns. GST-FE65 PTB1 bait pulled down 12, 27, and 0.7% of ARF6, ARF6 T27N, and ARF6 Q67L from the corresponding input lysates, respectively. Control pulldowns using GST-GGA3 as bait were performed in which the bait preferably binds to ARF6 Q67L mutant. EV-transfected cell lysates are included in panels **A** and **B** to demonstrate overexpression of ARF6. **C)** *E. coli*-expressed GST-FE65 PTB1 was used as baits in pulldown assays from myc-tagged ARF6-transfected lysates incubated with either GDP or GTP γ S (to load either GDP or GTP onto ARF6). ARF6 was detected by anti-myc 9B11. Also shown is an ARF6 activation assay (bottom panels) to demonstrate activation of ARF6 (increased binding to GST-GGA3 bait) in GTP γ S-treated lysates.

Figure 3.

FE65 stimulates both endogenous ARF6 and Rac1 activation. *A, B*) FE65 stimulates ARF6 activation. CHO cells were either transfected with EV or FE65 (*A*), or treated with either control (Ctrl) or FE65 siRNAs (*B*). Endogenous active ARF6 was pulled down from the transfected cell lysates using GST-GGA3-PBD bait (supplied in the active ARF6 pulldown kit; Cell Biolabs) that interacts specifically with ARF6-GTP. ARF6-GTP bound to the bait was analyzed on immunoblots. Immunoblots showing FE65, ARF6, and α -tubulin levels as a loading control in the cell lysates are also shown. FE65 was detected using anti-FE65 E20, ARF6 using anti-ARF6 6ARF01 and anti- α -tubulin DM1A. Overexpression of FE65 stimulates (*A*, top panel) while siRNA knockdown of FE65 inhibits (*B*, top panel) ARF6 activation. Longer exposures revealed the presence of ARF6 in the GGA3 pulldowns in control transfected cells in panel *A*. *C, D*) FE65 stimulates Rac1 activation. CHO cells were either EV transfected or transfected with FE65 (*C*) or treated with either control or FE65 siRNAs (*D*). Endogenous active Rac1 was pulled down from the transfected cell lysates using GST-PAK1 PBD bait [supplied in the Rac1 activation assay (ThermoScientific), which binds Rac1-GTP]. Rac1-GTP bound to the bait was analyzed on immunoblots using anti-Rac1 23A8. Immunoblots showing FE65, Rac1, and α -tubulin levels as a loading control in the cell lysates are also shown. Overexpression of FE65 stimulates (*C*, top panel) while knockdown of FE65 inhibits (*D*, top panel) Rac1 activation. *E*) Rac1 activation by FE65

requires ARF6. CHO cells were treated with control or ARF6 siRNAs and transfected with either EV or FE65. Rac1 activation assays were then performed. Top panel: immunoblot of the amounts of Rac1 in the PAK1 PBD pulldown assays. Also shown are immunoblots for FE65, ARF6, Rac1, and α -tubulin levels as a loading control in the cell lysates. Overexpression of FE65 did not trigger Rac1 activation in ARF6 knockdown cells (top panel, lane 2 vs. lane 4).

Figure 4. ARF6 is present in perinuclear regions and processes in developing neurons and colocalizes with FE65 in growth cones. Rat cortical neurons were immunostained at DIV2 for endogenous ARF6, FE65, and actin (via AlexaFluor-546 labeled phalloidin) and nuclei labeled using DAPI. *A*) Perinuclear and neurite labeling of ARF6. *B*) FE65 and ARF6 are present in both the cell bodies and processes but show a high level of colocalization in growth cones. Zoomed area of box with growth cone is shown. Scale bars = 10 μm.

Figure 5.

FE65 and ARF6 both stimulate neurite outgrowth, but the effect of FE65 is lost in the absence of ARF6. A–C) Rat cortical neurons were transfected with EGFP as a cell morphology marker and different combinations of EV control plasmid, FE65, FE65 Δ PTB1, FE65 Δ PTB2, ARF6, and either control, FE65, or ARF6 siRNAs as indicated. All transfections received the same amounts of DNA. The length of the longest neurite was then determined 24 h later. Bar charts show fold changes in mean neurite length for the longest neurite. Also shown are representative images of the different transfected cells. A) FE65 and ARF6 but not FE65 Δ PTB1 stimulate neurite outgrowth. On the other hand, FE65 Δ PTB2 induces a small increase in neurite extension. B) Knockdown of ARF6 inhibits neurite outgrowth, and this effect is not influenced by overexpression of FE65. C) Knockdown of FE65 inhibits neurite outgrowth, but this effect is rescued by overexpression of ARF6. Data were obtained from 40 cells/transfection, and the experiments were repeated 3 times. Error bars = SD. Scale bars = 10 μ m. * P < 0.001. D) Immunoblot demonstrating siRNA knockdown of FE65 and ARF6; also shown is an immunoblot for actin as a loading control.

Figure 6.

Rac1 is indispensable for FE65 and ARF6 stimulation of neurite outgrowth. *A, B*) Stimulation of neurite outgrowth by FE65 and ARF6 is abrogated in cells coexpressing a dominant-negative Rac1 (*A*) or transfected with Rac1 siRNA (*B*). *A*) Rat cortical neurons were transfected with EGFP and either EV, FE65 + ARF6, FE65 + ARF6 + Rac1, FE65 + ARF6 + N17Rac1 (dominant-negative Rac1), or V12Rac1 (constitutively active Rac1). *B*) Rat cortical neurons were transfected with EGFP and either EV, FE65 + ARF6 + control siRNA, and FE65 + ARF6 + Rac1 siRNA. *C*) Immunoblot shows siRNA knockdown of

Rac1. Constitutively active Rac1 stimulates neurite outgrowth. Rat cortical neurons were transfected with EGFP and either EV, V12Rac1 + control siRNA, V12Rac1 + FE65 siRNA, or V12Rac1 + ARF6 siRNA. In panels A–C, the length of the longest neurite was determined 24 h later. D) Effect of constitutively active Rac1 on neurite outgrowth is suppressed in the presence of CytoD. Rat cortical neurons were transfected with EGFP and either EV or V12Rac1. Neurons were treated with either DMSO or 0.25 $\mu\text{g/ml}$ CytoD for 24 h. Length of the longest neurite was then measured. Bar charts show fold changes in mean neurite length for the longest neurite. Also shown are representative images of the different transfected cells. Data were obtained from 40 cells/transfection, and the experiments were repeated 3 times. Error bars = SD . Scale bars = 10 μm . * $P < 0.001$.