Fe65-Elmo1 complex stimulates neurite outgrowth

Neuronal adaptor Fe65 stimulates Rac1-mediated neurite outgrowth by recruiting and activating Elmo1

Wen Li1, Ka Ming Vincent Tam 1, Wai Wa Ray Chan 1, Alex Chun Koon 1, Jacky Chi Ki Ngo2, Ho Yin Edwin Chan1 and Kwok-Fai Lau1

1School of Life Sciences, Faculty of Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR.

Running title: Fe65-Elmo1 complex stimulates neurite outgrowth

*To whom correspondence should be addressed: Dr. Kwok-Fai Lau, School of Life Sciences, Faculty of Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR. Email: kflau@cuhk.edu.hk. Phone no.: (852) 39431106. Fax no.: (852) 26037246

Keywords: ADP-ribosylation factor 6, Amyloid β A4 precursor protein-binding family B member 1, Guanine nucleotide exchange factor, neurite outgrowth, Ras-related C3 botulinum toxin substrate 1

ABSTRACT

Neurite outgrowth is a crucial process in developing neurons for neural network formation. Understanding the regulatory mechanisms of neurite outgrowth is essential for developing strategies to stimulate neurite regeneration after nerve injury and in neurodegenerative disorders. Fe65 is a brain-enriched adaptor that stimulates Rac1-mediated neurite elongation. However, the precise mechanism by which Fe65 promotes the process remains elusive. Here, we show that Elmo1, a subunit of Elmo1-DOCK180 bipartite Rac1 GEF, interacts with the Fe65 N-terminal region. Overexpression of Fe65 and/or Elmo1 enhances whereas knockdown of Fe65 or Elmo1 inhibits neurite outgrowth and Rac1 activation. The effect of Fe65 alone or together with Elmo1 is attenuated by an Fe65 double mutation that disrupts Fe65-Elmo1 interaction. Notably, Fe65 is found to activate Elmo1 by diminishing Elmo1 intramolecular autoinhibitory interaction and to promote the targeting of Elmo1 to the plasma membrane where Rac1 is activated. We also show that Fe65, Elmo1 and DOCK180 form a tripartite complex. Knockdown of DOCK180 reduces the stimulatory effect of Fe65-Elmo1 on Rac1 activation and neurite outgrowth. Thus, we identify a novel mechanism that Fe65 stimulates Rac1-mediated neurite outgrowth by recruiting and activating of Elmo1.

INTRODUCTION

Fe65 is a brain-enriched adaptor protein with several protein interaction domains including a WW domain and two C-terminal PTB domains (Fig. 1A). It is generally believed that Fe65 acts as a “scaffold” protein to recruit various interactors to form a functional complex. Indeed, a number of Fe65 interacting proteins (FIPs) are identified for its conserved domains. These findings have provided insights into the biological functions of Fe65. For instance, Fe65, via its PTB2 domain, is shown to interact with Alzheimer’s disease amyloid precursor protein (APP) and to alter APP processing. Moreover, Fe65 PTB1 domain is found to interact with the small GTPase ARF6 (reviewed by (1,2)). Additionally, Fe65 is implicated in neurodevelopment. Fe65 knockout mice are shown to have significant impairment in hidden-platform acquisition ability and reversal learning (3). Increased neonatal mortality and abnormal circling behavior are reported for Fe65/Fe65L1 double knockout mice. In fact, aberrant laminin organization of meningeal fibroblasts and axonal projection and pathfinding impairments in several brain regions have been observed in the mice (4). Moreover, neuromuscular junction abnormalities and LTP impairments are observed in the mice (5). We have also shown that Fe65 stimulates neurite outgrowth, an essential process for neural network formation, by activating ARF6-Rac1 signaling (6). Since Fe65 does not possess any guanine

...
nucleotide exchange factor (GEF) catalytic activity, the underlying mechanisms that FE65 stimulates the signaling pathway remain elusive. Nevertheless, the above findings underscore FE65 plays significant developmental roles in the nervous system.

Despite a number of interactors for the conserved domains are identified, FE65 N-terminal region (NTR) binding partners have not been properly determined yet. As an adaptor, it is possible that FE65 NTR is also involved in protein recruitment. To this end, we performed yeast two-hybrid screen using FE65 NTR as “bait”, and identified engulfment and cell motility protein 1 (ELMO1) as a novel FE65 interactor.

ELMO1 is an ubiquitously expressed protein containing an N-terminal Ras binding domain (RBD), an ELMO inhibitory domain (EID), an ELMO domain, a PH domain, an ELMO autoregulatory domain (EAD) and a C-terminal PXXP motif (Fig. 1A) (reviewed by (7)). ELMO1 is reported to be involved in various processes including phagocytosis, cell migration, cancer metastasis, dendritic spine morphogenesis and lymphocyte migration (reviewed by (8) and (9-11)). Many of these processes require dynamic remodeling of the actin cytoskeleton. The effects of ELMO1 are attributed, at least in part, through Rac1, a member of Rho GTPases that regulate actin dynamics. Rac1 is activated by the guanine nucleotide exchange factors (GEFs) which promote the exchange of GDP to GTP in Rac1. By contrast, GTPase-activating proteins (GAPs) inactivate Rac1 by promoting GTP hydrolysis (reviewed by (12)). In cells, ELMO1 complexes with DOCK180 to form a bipartite GEF in cells awaiting for the yet to be identified activation signal(s) to mediate Rac1-dependent cytoskeleton remodeling (7,13,14). Here, we show that FE65 interacts with ELMO1 and to diminish ELMO1 intramolecular autoinhibitory interaction. Moreover, FE65-ELMO1 interaction plays a role in stimulating Rac1-mediated neurite outgrowth.

RESULTS

**FE65 interacts directly with ELMO1** — From a yeast two-hybrid screen of a mouse brain cDNA library using FE651-217 fragment as ‘bait’, a cDNA clone encoding ELMO1126-727 which contains part of the PH domain, EAD and the PX motif, was isolated. Their interaction in yeast was confirmed using β-galactosidase colony-lift filter assay (Fig. 1B).

To further analyze the FE65-ELMO1 interaction, bacterially expressed GST and GST-ELMO1126-727 fusion proteins were used as baits to pull down FE65 from transfected cells. In this assay, ELMO1126-727, but not GST, pulled down FE65 (Fig. 1C). We next tested the FE65-ELMO1 interaction by using immunoprecipitation assays from transfected cells. FE65 was transfected either alone or co-transfected with myc-tagged ELMO1. ELMO1 was then immunoprecipitated via the myc-tag. FE65 was detected in immunoprecipitates from the FE65+ELMO1 but not FE65 singly transfected control cells (Fig. 1D). To demonstrate that endogenous FE65-ELMO1 interaction, we immunoprecipitated ELMO1 from rat brain and probed the immunoprecipitate for FE65. As shown in Fig. 1E, both ELMO1 and FE65 were present in the immunoprecipitates.

The above biochemical assays indicate that FE65 interacts with ELMO1. However, they could not exclude the possibility that FE65-ELMO1 interaction is mediated by an intermediate molecule. To examine whether this interaction is direct, we incubated *E. coli* purified His-FE651-217 with *E. coli* purified GST or GST-ELMO1126-727 baits. As shown in Fig. 1F, His-FE651-217 could be pulled down by GST-ELMO1126-727 but not GST. Taken together, these results demonstrate that FE65 interacts directly with ELMO1.

Next, we determined the regions in FE65 and ELMO1 that mediate their interaction. In addition to FE651-217, we further prepared three FE65 NTR yeast “bait” constructs. Yeast cells were co-transformed with ELMO1126-727 prey construct and different FE65 NTR baits, and then grew on double and quadruple dropout SD agar plates. We found that the transformants could not grow on quadruple dropout plate when FE65 amino acids 1 to 60 was deleted (Fig. 1G Top panel). This suggests that the first sixty residues of FE65 are essential for FE65-ELMO1 interaction. We then determined the residue(s) in FE65 that is critical for mediating FE65-ELMO1 interaction by generating a number of FE65 mutants, and tested their abilities to interact with ELMO1 in co-immunoprecipitation assays. We found that FE65 K48 and R51 (FE65m) are crucial for FE65-ELMO1 interaction as the interaction was markedly reduced by FE65 K48A/R51A double mutation (Fig. 1H)
On the other hand, ELMO1626-680 could not interact with FE651-217 in yeast two-hybrid assay (Fig. 1G Lower panel). This suggests that amino acids 681 to 727 of ELMO1, which contain EAD and PXXP, are required for the interaction. ELMO1 M692 and E693 are two critical residues in EAD for mediating protein interaction, at least with ELMO1 EID (15). Therefore, we tested if the two residues are essential for FE65-ELMO1 interaction. As shown in Fig. 1G, ELMO1 M692A/E693A (ELMO1m) double mutation abolished the interaction between ELMO1626-727 and FE651-217 in the yeast two-hybrid system. We confirmed the importance of the two residues by co-immunoprecipitation assay in which FE65-ELMO1 interaction was markedly reduced by ELMO1m double mutation (Fig. 1I). Together, our result suggests that FE651-60 and ELMO1 EAD are required for FE65-ELMO1 interaction.

**FE65 and ELMO1 promote neurite outgrowth and Rac1 activation** — Previously, we have found that FE65 stimulates Rac1-dependent neurite outgrowth (6). Since ELMO1 also enhances Rac1 activation and is widely expressed in different cell types including neurons (Fig. 2A), we anticipated that FE65-ELMO1 interaction promotes neurite extension. To test this, two days *in vitro* (DIV) rat primary cortical neurons were transfected with FE65, ELMO1 and FE65+ELMO1 together with enhanced green fluorescent protein expressing plasmid (pEGFP-C1) which serves as morphology marker for the transfected neurons. The expressions of FE65 and/or ELMO1 were confirmed by both Western blotting and immunofluorescence analyses (Fig. 2B Top right and bottom). Length of the longest neurite from the growth cone tip to the periphery of the cell body of the transfected neurons were measured 24 hours post-transfection. This is a widely employed neurite measurement approach (see examples (6,16,17)). Similar to our previous report (6), overexpression of FE65, but not FE65m, stimulated neurite extension (Fig. 2B Top left). Notably, overexpression of ELMO1 also promoted neurite outgrowth (Fig. 2B). As we have shown that ELMO1 interacts with FE65 (Fig. 1) and FE65 is expressed in neurons (Fig. 2A), the effect of overexpression of ELMO1 on neurite outgrowth might be due to the transfected ELMO1 interacted with endogenous FE65 in the neurons. In fact, a more pronounced stimulatory effect was observed in FE65+ELMO1 co-transfected neurons, and such effect was not observed in FE65m+ELMO1(Fig. 2B). As FE65m could not interact with ELMO1 properly, our findings suggest that FE65-ELMO1 interaction could promote neurite outgrowth.

Rac1 is a key regulator of actin cytoskeleton dynamics that controls a number of developmental events in neurons including neurite outgrowth (see review (18)). We therefore investigated if FE65-ELMO1 interaction influences Rac1 activity. As shown in Fig. 3A, overexpression of FE65 or ELMO1 could activate Rac1. However, FE65m could not stimulate Rac1. Of note, Rac1 was strongly activated in FE65+ELMO1, but not FE65m+ELMO1, co-transfected cells (Fig. 3A). Since FE65m has reduced binding ability towards ELMO1, our findings suggest that FE65-ELMO1 plays a role in promoting Rac1 activation.

To further dissect the roles of FE65 and ELMO1 in neurite outgrowth, siRNA knockdown approach was employed. Akin to our previous finding (6), knockdown of FE65 reduced the basal neurite extension significantly (Fig. 2C). Similar effect was observed in ELMO1 knockdown neurons (Fig. 2C). These observations further suggest that both FE65 and ELMO1 are essential for neurite outgrowth. Intriguingly, the stimulatory effect of overexpression of ELMO1 on neurite outgrowth and Rac1 were markedly reduced in FE65 knockdown cells (Figs. 2D and 3B). This suggests that FE65 is required, at least in part, for the effects of ELMO1 on neurite outgrowth and Rac1 activation. On the other hand, overexpression of FE65 could not stimulate neurite growth in ELMO1 knockdown neurons (Fig. 2E), and its effect on Rac1 activation was also decreased markedly in ELMO1 knockdown cells (Fig. 3C). Collectively, our findings suggest that FE65 plays a role, at least in part, in stimulating Rac1-mediated neurite outgrowth by interacting with ELMO1.

**DOCK180 is required for FE65 and ELMO1-mediated Rac1 activation and neurite outgrowth** — As stated before, ELMO1 is a subunit of ELMO1-DOCK180 bipartite Rac1 GEF complex (13). We therefore determined if FE65 alters the GEF complex formation. To do this, FLAG-DOCK180 was precipitated by anti-FLAG M2 antibody from DOCK180+ELMO1 and DOCK180+ELMO1+FE65 transfected cell lysates. As shown in Fig. 4A, the amount of ELMO1 in both immunoprecipitates were similar. Notably, FE65
FE65-ELMO1 complex stimulates neurite outgrowth

was also detected in the immunoprecipitate from ELMO1+DOCK180+FE65 transfected cell lysate (Fig. 4A). Moreover, we were able to determine a tripartite complex of DOCK180, ELMO1 and FE65 at endogenous level by immunoprecipitating DOCK180 from rat brain lysate (Fig. 4B). Taken together, both FE65 and DOCK180 can bind simultaneously to ELMO1 to form a tripartite complex.

Since DOCK180 is the catalytic subunit of ELMO1-DOCK180 bipartite Rac1 GEF complex (13), we tested if DOCK180 is essential for FE65-mediated Rac1 activation. Similar to our previous observation, overexpression of FE65 activated Rac1 (Fig. 4C). However, the effect of FE65 on Rac1 activation was reduced significantly in DOCK180 knockdown cells (Fig. 4C). Similarly, knockdown of DOCK180 decreased the effect of ELMO1 on Rac1 activation (Fig. 4C). The combined effect of FE65 and ELMO1 on Rac1 was also markedly lowered in DOCK180 knockdown cells (Fig. 4C).

We also determined the effect of DOCK180 knockdown on FE65-ELMO1-mediated neurite extension. As shown in Fig. 4D, knockdown of DOCK180 reduced the stimulating effects of FE65, ELMO1 and FE65+ELMO1 on neurite outgrowth. Together, our results suggest that DOCK180 is required, at least in part, for FE65-ELMO1-mediated Rac1 activation and neurite outgrowth.

**FE65 and ELMO1 co-localize on the plasma membrane and neuronal growth cones** — In order to interact in cells, FE65 and ELMO1 must be located within the same subcellular compartment. Since Rac1 is mainly activated on the plasma membrane (19), and both FE65 and ELMO1 stimulate Rac1, therefore we determined if FE65 and ELMO1 are expressed on the plasma membrane. Plasma membrane was prepared from both FE65+ELMO1 co-transfected cells and primary cortical neurons by using a Qproteome Plasma Membrane Protein Kit (Qiagen). Approximately 30% of plasma membrane elution and 1% total lysate (as size control) were subjected to immunoblotting for FE65, ELMO1 and various subcellular compartment markers. We found that both FE65 and ELMO1 were detected in the plasma membrane elutions from FE65+ELMO1 co-transfected cells (Fig. 5A) and primary cortical neurons (Fig. 5B).

The above biochemical plasma membrane isolation revealed the presence of both FE65 and ELMO1 in the plasma membrane elutions, we further analyzed if the two proteins are co-localized in cells by confocal microscopy. In fact, FE65 and ELMO1 were found co-localized in the perinuclear region of FE65+ELMO1 co-transfected cells. Noteworthy, a proportion of ELMO1 and FE65 were observed co-localized on the plasma membrane (Fig. 5C). We also determined if there was significant overlap in the distribution of the two proteins on plasma membrane by intensity correlation analysis (ICA) (6,20,21). The reporting intensity correlation quotient (ICQ) is statistically testable. For random staining, ICQ=0; for dependent staining (co-localization), 0<ICQ<0.5; for segregated staining, -0.5<ICQ<0. ICAs revealed a significant co-localization of FE65 and ELMO1 on the plasma membrane (Mean ±SEM ICQ= 0.15±0.01, p<0.001; n=40).

In rat cortical neurons, both distinct and overlapping staining were detected (Fig. 5D), in particular at the cell bodies. In fact, ICA at the cell bodies indicated a moderate covariance (ICQ=0.054±0.02; p<0.001; n=40). Since Rac1 is a regulator of actin cytoskeletal dynamics of growth cones at the tip of growing neurites (18,22,23), and FE65 potentiates the effect of ELMO1 on Rac1 activation, we therefore also enquired whether FE65 and ELMO1 co-localize in the growth cones of neurons. Akin to previous reports (6,24), FE65 was observed at the growth cones. Importantly, ELMO1 immunostain signal was overlapped significantly with FE65 in the growth cones (ICQ= 0.26±0.01; p<0.001; n=40) (Fig. 5D).

**FE65 releases ELMO1 autoinhibitory conformation and promotes plasma membrane targeting of ELMO1** — ELMO1 is shown to adopt a closed autoinhibitory conformation via intramolecular interaction between its N-terminal EID and C-terminal EAD that plays a role in regulating Rac1 signaling (15). It is proposed that the relief of ELMO1 autoinhibition is a prerequisite for targeting ELMO1-DOCK180 complex to the plasma membrane. Moreover, although yet to be identified, ELMO1 C-terminal interactors are suggested to play a role in Rac1 signaling by releasing ELMO1 autoinhibition constraints (7,13). Since our data reveals that FE65 interacts with the C-terminal of ELMO1, we therefore tested if FE65 influences ELMO1 EID-EAD interaction. Mammalian
expression constructs of myc-ELMO1, and GST-ELMO1 were transfected to the cells with empty vector (EV), FE65 or FE65m. GST-ELMO1 was captured from the cell lysates by glutathione-Sepharose 4B resin. Similar to the previous report, GST-ELMO1 bound to ELMO1 in the pull-down assay. Intriguingly, GST-ELMO1 could not pull down ELMO1 when co-transfected with FE65 but not FE65m mutant (Fig. 6A). Since FE65m interacts less with ELMO1 (Fig. 6A bottom panel and Fig. 1H), our data may suggest that FE65 interferes ELMO1 EID-EAD interaction by competing with ELMO1 N-terminal EID for binding to the C-terminal EAD.

To confirm FE65 disrupts ELMO1 autoinhibition conformation, proximity ligation assays (PLAs) were performed for ELMO1 and ELMO1 in cells. Mammalian expression constructs for myc-ELMO1 and FLAG-ELMO1 were transfected into the cells with EV, FE65 or FE65m. As shown in Fig. 6B, fluorescence PLA signals were observed in cells transfected with ELMO1 and ELMO1 which suggests that the two ELMO1 fragments interacted in cells. Upon co-transfection with FE65, but not FE65m, the number of PLA signals was significantly reduced in cells. No detectable signal was found in control PLAS (Fig. 6C). This observation further suggests that FE65 could interfere ELMO1 autoinhibitory conformation.

It is proposed that ELMO1 activation is a prerequisite for its plasma membrane targeting and Rac1 activation there. Thus, we tested whether FE65 alters plasma membrane recruitment of ELMO1. Intriguingly, knockdown of FE65 reduced the amount of both transfected and endogenous ELMO1 in the plasma membrane elutions (Figs. 7A and 7B). Similarly, knockdown of FE65 decreased the number of cells with ELMO1 plasma membrane localization significantly (Fig. 7F). Moreover, FE65m decreased the amount of ELMO1 in the plasma membrane elution as compared to the wildtype counterpart (Fig. 7C). These observations may indicate that FE65-ELMO1 interaction is required, at least in part, for targeting of ELMO1 to the plasma membrane. On the other hand, knockdown of ELMO1 did not have significant effect on the plasma membrane localization of FE65 in both biochemical plasma membrane isolation (Figs. 7D and 7E) and immunofluorescence analyses (Fig. 7G). Collectively, our findings suggest that FE65 interacts with ELMO1 to release ELMO1 autoinhibition constraints and facilitates the targeting of ELMO1 to the plasma membrane to stimulate Rac1 signaling.

**DISCUSSION**

In the present study, ELMO1 is identified as a novel interactor for the neuronal adaptor protein FE65. A number of interactors are reported for the conserved domains of FE65. To our knowledge, ELMO1 is the first reported FE65 NTR interactor. In addition to FE65, two closely related isosforms, FE65L1 and FE65L2 are identified. Even though functional redundancy between FE65 proteins is suggested, there is evidence that FE65 may have some specific biological roles. For instance, only FE65 is capable to mediate FE65-ACD transactivation but not the other two isosforms (reviewed by (25)). Moreover, learning and memory deficits are observed in FE65 single knockout mice which suggest that FE65L1 and FE65L2 may not be able to fully compensate some unique FE65 brain functions (3). Noteworthy, all three FE65 proteins contain a WW and two PTB domains, and their structural divergence occurs primarily in their NTR. Thus, FE65 NTR may be required for its unique functions. In fact, emerging evidence reveals that FE65 NTR has distinct cellular roles. For instance, an N-terminal truncated FE65 product, named p65FE65, has been identified, and the truncated form has a stronger interaction with several FE65 interactors than the full-length counterpart. Moreover, the truncated FE65 has a more potent effect on suppression of sAPPα secretion and reduced transactivation capability (26). The identification of ELMO1 as FE65 NTR interactor provide evidence that FE65 NTR could also function in recruiting proteins for modulating FE65 functions.

Previously, we have shown that FE65 stimulates Rac1 activation (6). Since it does not possess any GEF activities, the exact mechanism(s) by which FE65 promotes Rac1 signaling remains elusive. Intriguingly, we show here that FE65 interacts directly with ELMO1, a regulatory subunit of the ELMO1-DOCK180 Rac1 GEF complex, and knockdown of either ELMO1 or DOCK180 decreases the stimulatory effect of FE65 on Rac1 activation. These suggest that FE65 activates Rac1, at least in part, via ELMO1-DOCK180 Rac1 GEF.

Here, we also show that M692 and E693 within ELMO1 EAD are essential for FE65-
ELMO1 interaction in both Y2H and co-immunoprecipitation assays. The two residues are also crucial for mediating ELMO1 EID-EAD intramolecular interaction (15). These suggest that both FE65 and ELMO1 EID bind to a similar region on the ELMO1 EAD. Noteworthy, ELMO1 adopts an inactive closed conformation by its EID-EAD interaction at basal state, and release of such constraints is required for proper functioning of DOCK180 in Rac1 activation (15). Although it is long proposed that ELMO1 interactors play a part in relieving ELMO1 autoinhibition (reviewed by (7,13)), such molecule was yet to be identified. Our findings reveal that FE65 is an interactor of ELM01 EAD to diminish ELMO1 EID-EAD intramolecular inhibitory interaction. Of note, overexpression of either FE65 or ELMO1 also stimulates Rac1 activation and neurite outgrowth. This may due to the exogenous proteins interact with the endogenous partners as both FE65 and ELMO1 are expressed in the cells used in this study (Fig. 2A). As mentioned above, once the two proteins interact, FE65 releases ELMO1 autoinhibition conformation. The FE65-ELMO1 complex becomes “active” for the downstream activities such as Rac1 activation. Hence, it is essential to identify the upstream mechanism(s) that regulates FE65-ELMO1 interaction. It is reported that phosphorylation modulates the interactions between FE65 and its interactors (27-29). Therefore, it is possible that phosphorylation of FE65 and/or ELMO1 regulates their interaction and thereby FE65-ELMO1 complex activation.

In addition to ELMO1, FE65 has been shown to bind to ARF6, a small GTPase implicated in various neuronal processes including axonal elongation (6,30,31). The effects of ARF6 are attributed, at least in part, by activation of Rac1. However, the exact mechanism by which ARF6 stimulates Rac1 remains unclear. Of note, ARF family members have been shown to form multimeric complexes to activate various biological events. For example, a multimeric complex contains clathrin, ARF1, AP1 and CYFIP is reported to play a significant regulatory role in clathrin-AP1-coated carrier biogenesis (32). Additionally, several GTPases and their GAPs complex with ARF4 for the ciliary-trafficking of polycystin-1(33). Although ARF6 activation is reported to stimulate Rac1 signaling through ELMO1-DOCK180 complex (34), the linkage between ARF6 and ELMO1-DOCK180 complex was unknown. Therefore, it is worth investigating if FE65 functions as a link to orchestrate ARF6 and ELMO1-DOCK180 for stimulating Rac1 signaling.

Noteworthy, deregulation Rac1, a well-known Rho family small GTPase, has been implicated in a variety of diseases including, but not limited to, cancer, cardiovascular diseases and neurodegenerative disorders (35). For instance, defective Rac1 signaling is associated with Alzheimer’s disease and motor neuron disease (36-38). These diseases are characterized by synaptic loss and dysfunction. Of note, FE65 has been implicated in synaptic functions (5,6,39,40), and reduced FE65 expression is observed in both Alzheimer’s disease and motor neuron disease (41,42). Signaling pathways that regulate embryonic neurite development are conserved in adult for controlling synaptic plasticity including Rac1 signaling (43). Since we show here that FE65 regulates Rac1 activity by recruiting ELMO1, dysfunction of FE65 may play a role in the pathogenesis of Alzheimer’s disease and motor neuron disease.

Increasing evidence suggests roles of FE65 in cancer. For example, FE65 expression is reported to be increased in thyroid carcinomas which could stimulate cancer cell growth by promoting sAPP generation (44,45). Moreover, FE65 has been shown to enhance the transcriptional activity of the estrogen receptor α (ERα) and to stimulate the breast cancer cell growth (46). Additionally, it has been reported that FE65 suppresses the migration and invasion of ERα negative breast cancer cells (47). In addition to neuronal functions, our finding opens an avenue for studying the association between FE65, Rac1 signaling and carcinogenesis which may lead to novel therapeutic targets for intervention.

Together, we show that FE65 interacts with ELMO1-DOCK180 to stimulate Rac1-mediated neurite outgrowth by activating ELMO1 (Fig. 8).

**EXPERIMENTAL PROCEDURES**

**Plasmids** — Mammalian expression constructs for wild-type FE65 and myc-tagged FE65 were as described (48-50). FE65 K48A/R51A (FE65m) mutant was generated by using QuiikChange II site-directed mutagenesis kit (Agilent Technologies). The mammalian expression construct of myc-tagged ELMO1,
FLAG-tagged ELMO1 and FLAG-tagged DOCK180 are gifts from Prof Jean-François Côté (51), Prof Kodi S. Ravichandran (52) and Prof Michiyuki Matsuda (53), respectively. Yeast bait constructs of FE65 contain residues 1-217, 1-95, 1-60 and 61-217 were generated by subcloning the corresponding FE65 cDNA into pGBK7T. Myc-tagged ELMO1,315 was created by introducing a stop codon after ELMO1 residue 315 in myc-tagged ELMO1 construct using QuikChange II site-directed mutagenesis kit. FLAG-tagged ELMO1315,727 and GST-tagged ELMO1315-727 were generated by subcloning the corresponding cDNA into pCMV-Tag2B (Agilent Technologies) and pCloneG-ST (6), respectively. Yeast prey constructs of ELMO1 contain residues 626-727 and 626-680 were generated by subcloning the corresponding ELMO1 cDNA into pGADT7-RecAB. Both mammalian and yeast constructs for ELMO1 M692A/E693A mutant were generated by mutagenesis. The bacterial GST fusion protein construct of ELMO1 626-727 was prepared by subcloning of the ELMO1 corresponding cDNA into pGEX-6P-1 (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Antibodies — Rabbit anti-FE65 was as described (27), goat anti-FE65 (E20) was obtained from Santa Cruz and mouse anti-FE65 (4H324) was from Abcam. Rabbit anti-ELMO1 (PA5-28406) was from ThermoFisher Scientific. Goat (N-20) and mouse (B-7) anti-ELMO1 were purchased from Santa Cruz Biotechnology. Anti-Calreticulin (D3E6), mouse (9B11) and rabbit (71D10) anti-αtubulin antibodies were purchased from Cell Signaling Technology. Anti-polyHistidine (HIS-1), anti-GST, anti-FLAG (M2), anti-Pan-Cadherin (C1821) and β-COP (G6160) were purchased from Sigma. Anti-Rac1 (23A8) was obtained from Millipore. Anti-ARF6 (3A-1), anti-DOCK180 (C-19) and anti-αtubulin antibody (DM1A) were purchased from Santa Cruz Biotechnology. Anti-VDAC1 (ab15895) and anti-β-tubulin antibody (ab6046) were obtained from Abcam. Anti-GFP (JL-8), anti-GAPDH (AM4300) and anti-EAA1 antibody (610465) were purchased from Clontech, Ambion and BD Transduction Lab, respectively. Mouse and goat IgG controls were purchased from ThermoFisher Scientific.

Yeast two-hybrid system — Yeast two-hybrid screen of mouse brain Mate and Plate cDNA library (Clontech) was performed essentially as described previously using the cDNA which encodes for the human FE65 amino acids 1-217 as bait (6). β-galactosidase colony-lift filter assays were performed essentially as described previously (54).

For mapping the interacting regions, various pGBK7-Fe65 bait and pGADT7-ELMO1 prey constructs were co-transformed into yeast Y2H Gold, and then grew on double (-Leu/-Trp) and quadruple (-Ade/-His/-Leu/-Trp) dropout SD agar plates.

Cell culture and transfection — CHO, HEK293, and E18 primary rat cortical neurons were prepared and cultured as described previously (6,54).

For plasmid transfection, CHO and HEK293 cells were transfected with X-tremeGENE™ 9 (Roche), and rat cortical neurons were transfected with Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturers’ instructions and as previously described (6). Accell siRNAs and regular siRNAs were obtained from GE Dharmacon. siRNAs were transfected to CHO and HEK293 by using Lipofectamine RNAiMAX (ThermoFisher Scientific).

Protein binding assays — The GST and GST-fusion proteins were expressed in E. coli BL21 and captured by glutathione-Sepharose 4B resin according to the manufacturer’s instructions (GE Healthcare Biosciences). The expressed proteins were used as “baits” in GST pull-down assays as described (6,27,54).

Co-immunoprecipitation assays were performed essentially as described previously from transfected cell and rat brain lysates (6,27,54).

Direct binding assays were performed as described previously (6,54). In brief, GST and GST-ELMO1626-727 were expressed and purified from E. coli. The GST baits were used to pull down purified His-FE651-217. The protein complexes were captured by glutathione-Sepharose 4B resin, and then analyzed by SDS/PAGE and Western blotting.

Rac 1 activation assays — Rac1-GTP levels in cells were determined by using a Rac1 activation assay kit (Thermo Scientific) as described previously (6). The relative amount of Rac1-GTP in PAK-PBD pull-downs was expressed as a densitometric ratio of Rac1-GTP/total Rac1.

Densitometric analysis of Western blots — Densitometric analysis of Western blots was performed by C-DiGit blot scanner (LI-COR) and analyzed by LI-COR® Image Studio™ Software. Data was obtained from at least three independent experiments.
**Immunofluorescence studies and neurite length measurements** — Immunofluorescence staining and neurite length measurement were performed as described previously (6,54). Three independent experiments, with at least 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 (National Institutes of Health) with NeuronJ plugin (55). For colocalization study, intensity correction analyses were performed as described using Image J with JACoP plugin (6,20,21). Three independent experiments, with at least 40 cells were analyzed. Statistical analyses were performed using one-way ANOVA test with Bonferroni post hoc test. Differences were considered significant at p<0.05.

**Plasma membrane isolation** — Plasma membrane was isolated from cells by using a Qproteome Plasma Membrane Protein Kit (Qiagen). Briefly, cells were harvested in ice-cold PBS and then washed with 1X lysis buffer PM for two times. Cell pellets were lysed in 1X lysis buffer PM with protease inhibitors and lysis solution PL at 4°C for 15 min. The lysates were then cleared by centrifugation at 4°C, 450 g for 5 min. Supernatants were incubated with binding ligand PBL and Strep-Tactin Magnetic Beads at 4°C for 1 hour. The magnetic beads were washed with lysis buffer PM and followed by wash buffer twice. The isolated plasma membrane proteins were eluted by boiling in 1X SDS sample buffer, and 30% of plasma membrane elutions were subjected to Western blotting analysis for FE65 and ELMO1. The purity of the plasma membrane preparations was validated by probing the samples with various subcellular compartment marker antibodies. Approximately 1% of total lysates were loaded for size comparison.

**Proximity ligation assays** — Proximity ligation assay (PLA) was performed by using a Duolink In Situ Fluorescence kit (Sigma). In brief, HEK 293 cells were seeded on cover slips 24 hours before transfection. Mammalian expression constructs myc-ELMO1 and FLAG-ELMO1 were transfected into the cells with empty vector (EV), FE65 or FE65m. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton 24 hours post-transfection. After blocking with 5% FBS in PBS at 37°C for 1 hour, the cells were then incubated with goat anti-ELMO1 N-20 (1:100) and mouse anti-FLAG M2 (1:200) for 1 hour at room temperature to probe for myc-ELMO1 and FLAG-ELMO1, respectively. The cells were then washed three times with 1X Wash buffer A, and followed by incubation with Duolink In Situ PLA probe antimouse PLUS and anti-goat MINUS at 37°C for 1 hour in a humid incubator. After incubation, the cells were washed three times with 1X Wash buffer A and then one time with 0.01X Wash buffer B. The cover slips were mounted with Duolink In Situ mounting medium with DAPI. Images were captured by using an Olympus IX71 fluorescence microscope with an UPlanSAPo 60X water immersion objective. Fluorescence images were captured by a Nikon DS-Qi2 camera, and the fluorescence signals were quantified by the Object Count tool in Nikon NIS Elements. Cells were also stained with anti-β-tubulin as morphology marker.

**Statistical analyses** — All experiments were repeated at least three times. Statistical analyses were performed using one-way ANOVA tests with Bonferroni post-hoc test. Significance is indicated between different treatments as *p<0.001; **p<0.01; ***p<0.05; ns, not significant, p>0.05. Error bars shown are either SD or SEM.

**Acknowledgements**: We thank Prof Jean-François Côté (Montreal Clinical Research Institute), Prof Kodi S. Ravichandran (University of Virginia School of Medicine) and Prof Michiyuki Matsuda (Faculty of Medicine Kyoto University) for the mammalian expression construct of myc-tagged ELMO1, FLAG-tagged ELMO1 and FLAG-tagged DOCK180, respectively.

**Conflict of interest**: The authors declare that they have no conflicts of interest with the contents of this article.
**Footnotes:** This work was supported by funds from the Research Grants Council Hong Kong, Health and Medical Research Fund (Hong Kong), CUHK direct grant scheme, the United College endowment fund and the TUYF Charitable Trust.

The abbreviations used are: Aβ, amyloid-β; APP, amyloid precursor protein; ARF6, ADP-ribosylation factor 6; CHO, Chinese hamster ovary; DIV, days *in vitro*; DOCK180, Deducator of cytokinesis 180; ELMO1, Engulfment and Cell Motility 1; GAP, GTPase-activating protein; GEF, Guanine nucleotide exchange factor; GST, Glutathione S transferase; HEK, human embryonic kidney; PTB, Phosphotyrosine binding.

**Author contributions:** WL, KMVT, WWRC and ACK performed research. WL, JCKN, HYEC and KFL conceived the study, designed the experiments, analyzed the data and wrote the paper.
FE65-ELMO1 complex stimulates neurite outgrowth

REFERENCES

1. Minopoli, G., Gargiulo, A., Parisi, S., and Russo, T. (2012) Fe65 matters: New light on an old molecule. *IUBMB Life* **64**, 936-942
2. Chow, W. N., Cheung, H. N., Li, W., and Lau, K. F. (2015) FE65: Roles beyond amyloid precursor protein processing. *Cell Mol Biol Lett* **20**, 66-87
3. Wang, B., Hu, Q., Heam, M. G., Shimizu, K., Ware, C. B., Liggitt, D. H., Jin, L. W., Cool, B. H., Storm, D. R., and Martin, G. M. (2004) Isoform-specific knockout of FE65 leads to impaired learning and memory. *J Neurosci Res* **75**, 12-24
4. Guenette, S., Chang, Y., Hiesberger, T., Richardson, J. A., Eckman, C. B., Eckman, E. A., Hammer, R. E., and Herz, J. (2006) Essential roles for the FE65 amyloid precursor protein-interacting proteins in brain development. *EMBO J* **25**, 420-431
5. Strecker, P., Ludewig, S., Rust, M., Mundinger, T. A., Gorlich, A., Krachan, E. G., Mehrfeld, C., Herz, J., Korte, M., Guenette, S. Y., and Kins, S. (2016) FE65 and FE65L1 share common synaptic functions and genetically interact with the APP family in neuromuscular junction formation. *Sci Rep* **Uk** **6**
6. Cheung, H. N., Dunbar, C., Morotz, G. M., Cheng, W. H., Chan, H. Y., Miller, C. C., and Lau, K. F. (2014) FE65 interacts with ADP-ribosylation factor 6 to promote neurite outgrowth. *FASEB J* **28**, 337-349
7. Patel, M., Pelletier, A., and Cote, J. F. (2011) Opening up on ELMO regulation: New insights into the control of Rac signaling by the DOCK180/ELMO complex. *Small GTPases* **2**, 268-275
8. Ravichandran, K. S., and Lorenz, U. (2007) Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* **7**, 964-974
9. Li, H., Yang, L., Fu, H., Yan, J., Wang, Y., Guo, H., Hao, X., Xu, X., Jin, T., and Zhang, N. (2013) Association between Galpha12 and ELMO1/Dock180 connects chemokine signalling with Rac activation and metastasis. *Nat Commun* **4**, 1706
10. Kim, J. Y., Oh, M. H., Bernard, L. P., Macara, I. G., and Zhang, H. (2011) The RhoG/ELMO1/Dock180 signaling module is required for spine morphogenesis in hippocampal neurons. *J Biol Chem* **286**, 37615-37624
11. Stevenson, C., de la Rosa, G., Anderson, C. S., Murphy, P. S., Capece, T., Kim, M., and Elliott, M. R. (2014) Essential role of Elmo1 in Dock2-dependent lymphocyte migration. *J Immunol* **192**, 6062-6070
12. Ridley, A. J. (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* **16**, 522-529
13. Laurin, M., and Cote, J. F. (2014) Insights into the biological functions of Dock family guanine nucleotide exchange factors. *Genes Dev* **28**, 533-547
14. Komander, D., Patel, M., Laurin, M., Fradet, N., Pelletier, A., Barford, D., and Cote, J. F. (2008) An alpha-helical extension of the ELMO1 pleckstrin homology domain mediates direct interaction to DOCK180 and is critical in Rac signaling. *Mol Biol Cell* **19**, 4837-4851
15. Patel, M., Margaron, Y., Fradet, N., Yang, Q., Wilkes, B., Bouvier, M., Hofmann, K., and Cote, J. F. (2010) An evolutionarily conserved autoinhibitory molecular switch in ELMO proteins regulates Rac signaling. *Curr Biol* **20**, 2021-2027
16. Bryan, B., Kumar, V., Stafford, L. J., Cai, Y., Wu, G., and Liu, M. (2004) GEFT, a Rho family guanine nucleotide exchange factor, regulates neurite outgrowth and dendritic spine formation. *J Biol Chem* **279**, 45824-45832
17. Tudor, E. L., Perkinton, M. S., Schmidt, A., Ackerley, S., Brownlees, J., Jacobsen, N. J., Byers, H. L., Ward, M., Hall, A., Leigh, P. N., Shaw, C. E., McLoughlin, D. M., and Miller, C. C. (2005) ALS2/Alsin regulates Rac-PAK signaling and neurite outgrowth. *J Biol Chem* **280**, 34735-34740
18. Lundquist, E. A. (2003) Rac proteins and the control of axon development. *Curr Opin Neurobiol* **13**, 384-390
FE65-ELMO1 complex stimulates neurite outgrowth

19. Bustelo, X. R., Ojeda, V., Barreira, M., Sauzeau, V., and Castro-Castro, A. (2012) Rac-ing to the plasma membrane: the long and complex work commute of Rac1 during cell signaling. Small GTPases 3, 60-66

20. Li, Q., Lau, A., Morris, T. J., Guo, L., Fordyce, C. B., and Stanley, E. F. (2004) A syntaxin 1, Galphao(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. J Neurosci 24, 4070-4081

21. Stoops, E. H., Hull, M., and Caplan, M. J. (2016) Newly synthesized and recycling pools of the apical protein gp135 do not occupy the same compartments. Traffic 17, 1272-1285

22. Nikolic, M. (2002) The role of Rho GTPases and associated kinases in regulating neurite outgrowth. Int J Biochem Cell Biol 34, 731-745.

23. Etienne-Manneville, S., and Hall, A. (2002) Rho GTPases in cell biology. Nature 420, 629-635

24. Sabo, S. L., Ikin, A. F., Buxbaum, J. D., and Greengard, P. (2003) The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses in vitro and in vivo. J Neurosci 23, 5407-5415

25. McLoughlin, D. M., and Miller, C. C. (2008) The FE65 proteins and Alzheimer's disease. J Neurosci Res 86, 744-754

26. Hu, Q., Wang, L., Yang, Z., Cool, B. H., Zitnik, G., and Martin, G. M. (2005) Endoproteolytic cleavage of FE65 converts the adaptor protein to a potent suppressor of the sAPPalpha pathway in primates. J Biol Chem 280, 12548-12558

27. Lau, K. F., Chan, W. M., Perkinton, M. S., Tudor, E. L., Chang, R. C., Chan, H. Y., McLoughlin, D. M., and Miller, C. C. (2008) Dextras1 Interacts with FE65 to Regulate FE65-Amyloid Precursor Protein-dependent Transcription. J Biol Chem 283, 34728-34737

28. Chow, W. N., Ngo, J. C., Li, W., Chen, Y. W., Tam, K. M., Chan, H. E., Miller, C. C., and Lau, K. F. (2015) Phosphorylation of FE65 Serine-610 by serum- and glucocorticoid-induced kinase 1 modulates Alzheimer's disease amyloid precursor protein processing. Biochem J 470, 303-317

29. Lee, Y. S., Chow, W. N. V., and Lau, K. F. (2017) Phosphorylation of FE65 at threonine 579 by GSK3beta stimulates amyloid precursor protein processing. Sci Rep 7, 12456

30. Jaworski, J. (2007) ARF6 in the nervous system. Eur J Cell Biol 86, 513-524

31. Hernandez-Deviez, D. J., Roth, M. G., Casanova, J. E., and Wilson, J. M. (2004) ARNO and ARF6 regulate axonal elongation and branching through downstream activation of phosphatidylinositol 4-phosphate 5-kinase alpha. Mol Biol Cell 15, 111-120

32. Anitei, M., Stange, C., Parshina, I., Baust, T., Scheneck, A., Raposo, G., Kirchhausen, T., and Hoflack, B. (2010) Protein complexes containing CYFIP/Sra/PIR121 coordinate Arf1 and Rac1 signalling during clathrin-AP-1-coated carrier biogenesis at the TGN. Nat Cell Biol 12, 330-340

33. Ward, H. H., Brown-Glaberman, U., Wang, J., Morita, Y., Alper, S. L., Bedrick, E. J.,Gattone, V. H., 2nd, Deretic, D., and Wandinger-Ness, A. (2011) A conserved signal and GTPase complex are required for the ciliary transport of polycystin-1. Mol Biol Cell 22, 3289-3305

34. Santy, L. C., Ravichandran, K. S., and Casanova, J. E. (2005) The Dock180/Elmo complex couples ARNO-mediated Arf6 activation to the downstream activation of Rac1. J Biol Chem 280, 1749-1754

35. Marei, H., and Malliri, A. (2016) Rac1 in human diseases: The therapeutic potential of targeting Rac1 signaling regulatory mechanisms. Small GTPases, 1-25

36. Nadif Kasri, N., and Van Aelst, L. (2008) Rho-linked genes and neurological disorders. Pflugers Arch 455, 787-797

37. Zhao, L., Ma, Q. L., Calon, F., Harris-White, M. E., Yang, F., Lim, G. P., Morihara, T., Ubeda, O. J., Ambegaokar, S., Hansen, J. E., Weisbart, R. H., Teter, B., Frautschy, S. A., and Cole, G. M. (2006) Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease. Nat Neurosci 9, 234-242

38. Stankiewicz, T. R., and Linseman, D. A. (2014) Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration. Front Cell Neurosci 8, 314
FE65-ELMO1 complex stimulates neurite outgrowth

39. Masin, M., Kerschensteiner, D., Dumke, K., Rubio, M. E., and Soto, F. (2006) Fe65 interacts with P2X2 subunits at excitatory synapses and modulates receptor function. *J Biol Chem* **281**, 4100-4108

40. Nens, F. M., Neumann, M. H., Schrotter, A., Przyborski, A., Mastalski, T., Susdalzew, S., Loobetae, C., Helling, S., El Magraoui, F., Erdmann, R., Meyer, H. E., Uszkoreit, J., Eisenacher, M., Suh, J., Guenette, S. Y., Rohner, N., Kogel, D., Theiss, C., Marcus, K., and Muller, T. (2014) Amyloid beta a4 precursor protein-binding family B member 1 (FE65) interactomics revealed synaptic vesicle glycoprotein 2A (SV2A) and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) as new binding proteins in the human brain. *Mol Cell Proteomics* **13**, 475-488

41. Kajiwara, Y., Akram, A., Katsel, P., Haroutunian, V., Schmeidler, J., Beecham, G., Haines, J. L., Pericak-Vance, M. A., and Buxbaum, J. D. (2009) FE65 binds Teashirt, inhibiting expression of the primate-specific caspase-4. *PLoS One* **4**, e5071

42. Gomez-Pinedo, U., Villar-Quiles, R. N., Galan, L., Matias-Guiu, J. A., Benito-Martin, M. S., Guerrero-Sola, A., Moreno-Ramos, T., and Matias-Guiu, J. (2016) Immunonochemical Markers of the Amyloid Cascade in the Hippocampus in Motor Neuron Diseases. *Front Neurol* **7**, 195

43. Martinez, L. A., and Tejada-Simon, M. V. (2011) Pharmacological inactivation of the small GTPase Rac1 impairs long-term plasticity in the mouse hippocampus. *Neuropharmacology* **61**, 305-312

44. Pietrzik, C. U., Hoffmann, J., Stober, K., Chen, C. Y., Bauer, C., Otero, D. A., Roch, J. M., and Herzog, V. (1998) From differentiation to proliferation: the secretory amyloid precursor protein as a local mediator of growth in thyroid epithelial cells. *Proc Natl Acad Sci U S A* **95**, 1770-1775

45. Krause, K., Karger, S., Sheu, S. Y., Aigner, T., Kursawe, R., Gimm, O., Schmid, K. W., Dralle, H., and Fuhrer, D. (2008) Evidence for a role of the amyloid precursor protein in thyroid carcinogenesis. *J Endocrinol* **198**, 291-299

46. Sun, Y., Kasiappan, R., Tang, J., Webb, P. L., Quarni, W., Zhang, X., and Bai, W. (2014) A novel function of the Fe65 neuronal adaptor in estrogen receptor action in breast cancer cells. *J Biol Chem* **289**, 12217-12231

47. Sun, Y., Sun, J., Lungchukiet, P., Quarni, W., Yang, S., Zhang, X., and Bai, W. (2015) Fe65 Suppresses Breast Cancer Cell Migration and Invasion through Tip60 Mediated Cortactin Acetylation. *Sci Rep* **5**, 11529

48. Lau, K. F., McLoughlin, D. M., Standen, C. L., Irving, N. G., and Miller, C. C. (2000) Fe65 and X11β co-localize with and compete for binding to the amyloid precursor protein. *Neuroreport* **11**, 3607-3610

49. Perkinton, M. S., Standen, C. L., Lau, K. F., Kesavapany, S., Byers, H. L., Ward, M., McLoughlin, D. M., and Miller, C. C. (2004) The e-Abl tyrosine kinase phosphorylates the Fe65 adaptor protein to stimulate Fe65/amyloid precursor protein nuclear signaling. *J Biol Chem* **279**, 22084-22091

50. Cao, X., and Sudhof, T. C. (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**, 115-120.

51. Margaron, Y., Fradet, N., and Cote, J. F. (2013) ELMO recruits actin cross-linking family 7 (ACF7) at the cell membrane for microtubule capture and stabilization of cellular protrusions. *J Biol Chem* **288**, 1184-1199

52. Mauldin, J. P., Lu, M., Das, S., Park, D., Ernst, P. B., and Ravichandran, K. S. (2013) A link between the cytoplasmic engulfment protein Elmo1 and the Mediator complex subunit Med31. *Curr Biol* **23**, 162-167

53. Nishihara, H., Kobayashi, S., Hashimoto, Y., Ohba, F., Mochizuki, N., Kurata, T., Nagashima, K., and Matsuda, M. (1999) Non-adherent cell-specific expression of DOCK2, a member of the human CDM-family proteins. *Biochim Biophys Acta* **1452**, 179-187

54. Hao, C. Y., Perkinton, M. S., Chan, W. W., Chan, H. Y., Miller, C. C., and Lau, K. F. (2011) GULP1 is a novel APP-interacting protein that alters APP processing. *Biochem J* **436**, 631-639
FE65-ELMO1 complex stimulates neurite outgrowth

55. Meijering, E., Jacob, M., Sarria, J. C., Steiner, P., Hirling, H., and Unser, M. (2004) Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry* A 58, 167-176
FIGURE LEGENDS

FIGURE 1. FE65 interacts with ELMO1. (A) Schematic diagrams to show the various subdomains and mutants of FE65 and ELMO1 used in this study. Asterisks indicate the positions of mutations in FE65 and ELMO1. (B) FE65\(^{1-217}\) interacts with ELMO1\(^{626-727}\) in yeast two-hybrid system. pGBK7-Fe65\(^{1-217}\)+pGADT7, pGBK7-ELMO1\(^{626-727}\), pGBK7-Fe65\(^{1-217}\)+pGADT7-ELMO1\(^{626-727}\) and pGBK7-p53+pGADT7-T were transformed into the yeast strain Y2H Gold and grew on quadruple (-Asc/-His/-Leu/-Trp: QDO) dropout SD agar plates. Colony-lifts assays were performed for the transformants. Blue signals were observed in yeast transformed with pGBK7-Fe65\(^{1-217}\)+pGADT7-ELMO1\(^{626-727}\) and pGBK7-p53+pGADT7-T (positive control). (C) ELMO1 binds to FE65 in GST pull-down assays. E. coli expressed GST and GST-ELMO1\(^{626-727}\) were used as baits in pull-down assays from FE65 transfected cells. FE65 in the cell lysates and pull-downs were detected by using anti-FE65 E20 antibody (1:5000). Coomassie blue gel showed the GST and GST-ELMO1 baits. (D) FE65 and ELMO1 interact in immunoprecipitation assays from transfected cells. Immunoprecipitations were performed from CHO cells transfected with FE65 or FE65+myc-tagged ELMO1. ELMO1 was immunoprecipitated using anti-myc antibody 9B11. Immunoprecipitated FE65 and ELMO1 were detected by a goat anti-FE65 (1:5000) and a rabbit anti-myc (1:5000), respectively. (IgG) and (9B11) refer to the presence of mouse control IgG and myc antibody 9B11 in the immunoprecipitations, respectively. (E) FE65 interacts endogenously with ELMO1. ELMO1 was immunoprecipitated from rat brain lysate using mouse anti-ELMO1 B-7. Immunoprecipitated ELMO1 and FE65 were detected using a rabbit anti-ELMO1 antibody PA5-28406 (1:500) and goat anti-FE65 E20 antibody (1:1000), respectively. (IgG) and (B-7) refer to the presence of mouse control IgG and anti-ELMO1 in the immunoprecipitations, respectively. (F) FE65 interacts directly with ELMO1. E. coli expressed GST and GST-ELMO1\(^{626-727}\) were used to pull down purified His-tagged FE65\(^{1-217}\). Bottom panel shows recombinant bait proteins in the pull-downs; Top panel shows the pull-downs and the purified His-tagged FE65\(^{1-217}\). (G) FE65\(^{1-60}\) and ELMO1\(^{680-727}\) are essential for FE65-ELMO1 interaction. FE65\(^{1-217}\), FE65\(^{1-95}\), FE65\(^{61-217}\) and FE65\(^{1-60}\) were cloned into pGBK7. ELMO1\(^{626-727}\), ELMO1\(^{626-680}\) and ELMO1\(^{626-727}\) M692A/E693A (ELMO1\(^{m627-727}\)) were cloned into pGADT7. FE65 and ELMO1 constructs were co-transformed into yeast Y2H Gold. The transformants were grown on double (-Leu/-Trp; DDO) and quadruple (-Adr/-His/-Leu/-Trp; QDO) dropout SD agar plates. (H) FE65 K48 and R51 and (I) ELMO1 M692 and E693 are crucial residues for the interaction. Myc-tagged ELMO1 was immunoprecipitated from the transfected lysates using anti-myc 9B11. ELMO1 and FE65 in the immunoprecipitates were detected using rabbit anti-ELMO1 antibody (1:5000) and goat anti-FE65 antibody E20 (1:5000), respectively. (IgG) and (B-7) refer to the presence of mouse control IgG and anti-ELMO1 in the immunoprecipitations, respectively. Lysates and pull-down/IP samples in (C, D, E, F, H and I) were probed with anti-\(\alpha\)-tubulin DM1A antibody (1:5000) to illustrate lysate loading and pull-downs/IP specificity, respectively. FE65 and ELMO1 levels were measured by a C-Dig blot scanner (LI-COR) and analyzed by LI-COR® Image Studio™ Software. Data for graphs in (H and I) were obtained from independent experiments. n=3. *p<0.001. Results are means ± SD.

FIGURE 2. FE65-ELMO1 interaction stimulates neurite outgrowth. (A) Western bots to show the expression of FE65 and ELMO1 in different cell types. Approximately 10\(\mu\)g of lysate from E18 rat embryonic cortical neurons, CHO, SH-SY5Y and HEK293 cells were loaded. FE65 and ELMO1 were detected by anti-FE65 E20 (1:1000) and anti-ELMO1 B-7 (1:5000), respectively. (B, C, D, E) Rat primary cortical neurons were transfected with EGFP as a cell morphology marker and different combinations of FE65, and ELMO1 constructs, and either control, FE65 or ELMO1 siRNAs as indicated. All transfections received the same amounts of DNA. The length of the longest neurite was then determined 24 hours later. Bar charts show fold changes in mean neurite length for the longest neurite. Representative images of the different transfected cells are shown for (B). FE65-myc and myc-ELMO1 were stained with a rabbit anti-FE65 (1:5000) and mouse anti-ELMO1 B-7 (1:1000), respectively. Scale bar is 10\(\mu\)m. The expressions of EGFP and FE65-myc/myc-ELMO1 were also determined by Western blot analysis using anti-GFP JL-8 (1:5000) and anti-myc 9B11 (1:2500), respectively. \(\alpha\)-tubulin in the lysates was determined by anti-\(\alpha\)-tubulin DM1A antibody (1:5000). Both FE65 and ELMO1 enhance neurite outgrowth. More potent effects are observed in FE65+ELMO1, but not in FE65m+ELMO1,
co-transfected cells (B). Knockdown of either FE65 or ELMO1 reduces the basal neurite extension in the cortical neurons (C). Knockdown of FE65 reduces the effect of ELMO1 on neurite outgrowth (D). Knockdown of ELMO1 suppresses the effect of FE65 on neurite outgrowth (E). In (B, C, D and E), data was obtained from at least 40 cells per transfection and the experiments were repeated three times. *p<0.001; ***p<0.05. Error bars are SEM.

**FIGURE 3.** FE65-ELMO1 interaction activates Rac1. (A, B, C) CHO cells were transfected with the constructs empty vector control plasmid (EV), FE65, FE65m and ELMO1 constructs, and either control, FE65 or ELMO1 siRNAs as indicated. Active Rac1 was pulled down from the transfected cell lysates using GST-PAK1 PBD bait (supplied in the Rac1 activation assay kit (ThermoFisher Scientific)) which binds Rac1-GTP. Rac1-GTP bound to the bait was analyzed on immunoblots using anti-Rac1 23A8 (1:5000), FE65 E20 (1:5000), Rac1 23A8 (1:5000), ELMO1 B-7 (1:5000) and α-tubulin DM1A (1:5000) expression levels in the cell lysates are also shown. Both FE65 and ELMO1 enhances Rac1 activation. More potent effects are observed in FE65+ELMO1, but not FE65m+ELMO1, co-transfected cells (A). Knockdown of FE65 decreases the effect of ELMO1 on Rac1 activation (B). Knockdown of ELMO1 reduces the effect of FE65 on Rac1 activation (C). In (A-C), the relative Rac1-GTP level was expressed as a densitometric ratio of Rac1-GTP/total Rac1. Data for graphs were obtained from three independent experiments. *p<0.001; **p<0.01. Results are means ± SD.

**FIGURE 4.** DOCK180 is required for FE65 and ELMO1-mediated Rac1 activation and neurite outgrowth. (A) DOCK180, ELMO1 and FE65 interact in immunoprecipitation assays from transfected cells. Immunoprecipitations were performed from CHO cells transfected with DOCK180+ELMO1 and DOCK180+ELMO1+FE65. DOCK180 was immunoprecipitated using anti-FLAG against the FLAG tag on DOCK180. DOCK180 in the immunoprecipitates was detected by anti-DOCK180 C-19 (1:2500), and ELMO1, FE65 and α-tubulin were detected by anti-ELMO1 PA5-28406, anti-FE65 E20 and anti-α-tubulin DM1A, respectively. (IgG) and (M2) refer to the presence of mouse control IgG and anti-FLAG M2 in the immunoprecipitations, respectively. ELMO1 level in IPs was measured and analyzed as described in Fig. 1. The relative ELMO1 level in IP was expressed as a densitometric ratio of ELMO1 in IP/DOCK180 in IP. Data for graphs were obtained from three independent experiments. ns, p>0.05. Results are means ± SD. (B) DOCK180, ELMO1 and FE65 form complex at endogenous level. DOCK180 was immunoprecipitated from rat brain lysate using goat anti-DOCK180 C-19. The immunoprecipitates were analyzed for the presence of DOCK180, ELMO1, FE65 and α-tubulin by using anti-DOCK180 C-19 (1:1000), anti-ELMO1 PA5-28406 (1:500), anti-FE65 E20 (1:1000) and anti-α-tubulin DM1A (1:5000), respectively. (IgG) and (C-19) refer to the presence of goat control IgG and anti-DOCK180 C-19 in the immunoprecipitations, respectively. (C) CHO cells were transfected with the constructs empty vector control plasmid (EV), FE65 and ELMO1 constructs, and either control or DOCK180 siRNAs as indicated. Rac1 activation assays were performed as described in Fig. 3. Knockdown of DOCK180 reduces the effects of FE65, ELMO1 and FE65+ELMO1 on Rac1 activation. In (C), the relative Rac1-GTP level was expressed as a densitometric ratio of Rac1-GTP/total Rac1. Data for graphs were obtained from three independent experiments. *p<0.001. Results are means ± SD. (D) Rat primary cortical neurons were transfected with the constructs empty vector control plasmid (EV) and different combinations of FE65, ELMO1, control siRNA and DOCK180 siRNA as indicated for neurite outgrowth assay. Knockdown of DOCK180 suppresses the stimulatory effects of FE65, ELMO1 and FE65+ELMO1 on neurite outgrowth. The expressions of EGFP, FE65-myc/myc-ELMO1 and DOCK180 were determined by Western blot analysis using anti-GFP JL-8 (1:5000), anti-myc 9B11 (1:2500) and anti-DOCK180 C-19 (1:2500), respectively. α-tubulin in the lysates was determined by α-tubulin DM1A antibody (1:5000). Data was obtained from at least 40 cells per transfection and the experiments were repeated three times. *p=0.001. Error bars are SEM.
FE65-ELMO1 complex stimulates neurite outgrowth

**FIGURE 5.** Co-localization of FE65 and ELMO1 in transfected cells and neurons. Plasma membrane was isolated from (A) FE65 and ELMO1 co-transfected CHO cells and (B) rat primary cortical neurons by using a Qproteome Plasma Membrane Protein Kit (Qiagen). Approximately 1% of total lysate and 30% plasma membrane (PM) elutions were analyzed by immunoblotting by using anti-FE65 E20 (1:1000), anti-ELMO1 B-7 (1:500) and various subcellular compartment marker antibodies including Pan-Cadherin (1:2000), calreticulin (1:1000), β-COP (1:1000), EEA1 (1:1000), GAPDH (1:5000) and VADC1 (1:5000). (C) Immunofluorescent staining of COS7 cells transfected with ELMO1 and FE65-myc. ELMO1 and FE65-myc were stained by a goat anti-ELMO1 N-20 (1:100) and a mouse anti-myc antibody 9B11 (1:1000). Overlaid image is shown. Nuclei were labeled by 4’,6-diamidino-2-phenylindole (DAPI). Scale bar is 10μm. Similar to other reports, FE65 is observed in both cytoplasm and nucleus. ELMO1 is mainly located in the cytoplasm. The two proteins are mainly co-localized in the perinuclear region. A proportion of ELMO1 and FE65 are co-localized on the plasma membrane (pointed). Both FE65 and ELMO1 are expressed on the plasma membrane. (D) FE65 and ELMO1 are co-localized in growth cones. Rat cortical neurons were immunostained at DIV3 for endogenous FE65 and ELMO1 by a rabbit anti-FE65 (1:100) and a mouse anti-ELMO1 B-7 (1:100). Zoomed area of boxes with growth cones are shown. Scale bar is 10μm. In (C) and (D), two representative cells are shown.

**FIGURE 6.** FE65 releases ELMO1 autoinhibitory conformation. (A) FE65, but not FE65m, reduces the intramolecular interaction of ELMO1 EID and EAD in GST pull-down assays. GST-ELMO1 (1:315-727) and myc-ELMO1 (1:315-727) were transfected into cells with either EV, FE65 or FE65m. GST-ELMO1 (1:315-727) was pulled down from the cells lysates by glutathione-Sepharose 4B. The lysates and pull-downs were analyzed for GST-ELMO1 (1:315-727), myc-ELMO1 (1:315), FE65 and α-tubulin by immunoblotting using anti-GST, anti-myc, anti-FE65 and anti-α-tubulin as stated in Fig. 1, respectively. Data were obtained from three independent experiments. *p<0.001. Results are means ± SD. (B) FE65, but not FE65m, reduces the intramolecular interaction of ELMO1 in PLA. myc-ELMO1 (1:315-727) and FLAG-ELMO1 (1:315-727) were transfected into the cells with EV, FE65 or FE65m. Anti-ELMO1 N-20 (recognizes myc-ELMO1 (1:315) or anti-FLAG M2 (recognizes FLAG-ELMO1 (1:315-727)) were used as primary antibodies for the assays. Reduced ELMO1 (1:315-727) in ELMO1 (1:315-727) PLA signals were observed in the cells co-transfected with FE65 as compared with the cells co-transfected with EV or FE65m. Representative images are shown. Scale bar is 10μm. Cells were stained with β-tubulin (1:200) and DAPI as morphology and nucleus markers, respectively. The bar chart shows the relative PLA signal (fold to EV) in different transfections. Data were obtained from at least 60 cells per transfection and the experiments were repeated three times. *p<0.001. Error bars are SEM. (C) Control PLAs were performed by transfecting myc-ELMO1 (1:315-727) and FLAG-ELMO1 (1:315-727) to cells. PLAs were performed for either no antibody (No Ab), anti-ELMO1 N-20 or Anti-FLAG M2. No PLA signal was observed in all the samples. Cells were also stained with β-tubulin and DAPI as morphology and nuclear markers, respectively. Scale bar is 10μm.

**FIGURE 7.** FE65 is required for the plasma membrane targeting of ELMO1. Plasma membrane (PM) was isolated from CHO cells transfected with either (A) ELMO1+control siRNA or ELMO1+FE65 siRNA, (B) control siRNA or FE65 siRNA, (C) ELMO1+FE65 or ELMO1+FE65m, (D) FE65+control siRNA or FE65+ELMO1 siRNA or (E) control siRNA or ELMO1 siRNA. Knockdown of FE65 reduced the level of transfected (A) and endogenous (B) ELMO1 in the PM elutions. Overexpression of FE65m decreases the amount of ELMO1 in the PM elution (C). Knockdown of ELMO1 did not show any significant effect on the amounts of transfected (D) and endogenous (E) FE65 in the PM elutions. In (A-E), Pan-cadherin and GAPDH serve as plasma membrane and cytosol markers, respectively. 30% of plasma membrane elution of each treatment was analyzed as described in Fig. 5. ELMO1 level in the PM elution and total ELMO1 level in lysate were measured and analyzed as described in Fig. 5. The relative ELMO1 level in PM elution was expressed as a densitometric ratio of ELMO1 in PM/total ELMO1 in the lysate. Data for graphs were obtained from three independent experiments. *p<0.001; ns, p>0.05. Results are means ± SD. COS7 cells were transfected with (F) ELMO1+control siRNA or ELMO1+FE65 siRNA, (G) FE65+control siRNA or FE65+ELMO1 siRNA. Cells
in (F) and (G) were then immunostained for ELMO1 and FE65 as described Fig. 5. The number of cells with or without (F) ELMO1 and (G) FE65 PM were countered. Two representative cells were shown. Knockdown of FE65 and ELMO1 were confirmed by Western blot analysis. Three independent experiments were performed and at least 50 cells were countered in each experiment. Data were expressed as percentage of cells with (F) ELMO1 and (G) FE65 PM localization. *p<0.001; ns, p>0.05. Error bars are SD.

**FIGURE 8.** Schematic diagram illustrates the role of FE65-ELMO1 interaction. In growth cone, FE65 recruits ELMO1-DOCK180 bipartite Rac1 GEF complex, and to activate ELMO1 by disrupting its autoinhibitory closed conformation from between EID and EAD. Once activated, FE65-ELMO1-DOCK180 complex is targeted to the plasma membrane to promote Rac1 activation and thereby neurite outgrowth.
FE65-ELMO1 complex stimulates neurite outgrowth
FE65-ELMO1 complex stimulates neurite outgrowth

Figure 2

(A) Rat embryonic cortical neuron

(B) Fold change in mean neurite length

(C) Fold change in mean neurite length

(D) Fold change in mean neurite length

(E) Fold change in mean neurite length

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
FE65-ELMO1 complex stimulates neurite outgrowth

Figure 3
FE65-ELMO1 complex stimulates neurite outgrowth

Figure 4

(A) Lysates and IPs

(B) Relative ELMO1 level in IPs

(C) Ctrl siRNA and DOCK180 siRNA

(D) Fold change in mean neurite length

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
FE65-ELMO1 complex stimulates neurite outgrowth

**Figure 5**

(A) FE65 & ELMO1 Transfected Cortical Neurons 

| kDa | 1% of Total Lysates | 30% of PM Elutions | 30% of PM Elutions |
|-----|---------------------|--------------------|--------------------|
| 100 |                     |                    |                    |
| 70  |                     |                    |                    |
| 100 |                     |                    |                    |
| 170 |                     |                    |                    |
| 130 |                     |                    |                    |
| 55  |                     |                    |                    |
| 130 |                     |                    |                    |
| 100 |                     |                    |                    |
| 170 |                     |                    |                    |
| 40  |                     |                    |                    |
| 35  |                     |                    |                    |

(B) Pan-Cadherin (PM) Calreticulin (ER) β-COP (Golgi) EEA1 (Endosome) GAPDH (Cytosol) VDAC1 (Mitochondria)

(C) FE65 ELMO1 MERGE DAPI

(D)
Figure 6

(A) Lysates vs. Pull-downs

(B) ELMO1<sup>315-727</sup> + ELMO1<sup>315-727</sup>

(C) Control PLAs
ELMO1<sup>315-727</sup> + ELMO1<sup>315-727</sup>

No Ab Anti-ELMO1 Anti-FLAG

PLA signals

β-Tubulin

DAPI

Merge

Relative myc-ELMO1<sup>315-727</sup> level in pull-downs

ELMO1<sup>315-727</sup>/ELMO1<sup>1-315</sup>

1.2

0.8

0.6

0.4

0.2

0
FE65-ELMO1 complex stimulates neurite outgrowth

Figure 7

(A) ELMO1 + Ctrl siRNA  
(B) Ctrl siRNA  
(C) ELMO1 + FE65 siRNA

(D) FE65 + Ctrl siRNA  
(E) Ctrl siRNA

(F) Relative ELMO1 level in PM elutions

(G) Ctrl siRNA  
(ELMO1 siRNA)

Cells with ELMO1 PM localization (%)
FE65-ELMO1 complex stimulates neurite outgrowth
Neuronal adaptor FE65 stimulates Rac1-mediated neurite outgrowth by recruiting and activating ELMO1

Wen Li, Ka Ming Vincent Tam, Wai Wan Ray Chan, Alex Chun Koon, Jacky Chi Ki Ngo, Ho Yin Edwin Chan and Kwok-Fai Lau

*J. Biol. Chem.* *published online April 3, 2018*

Access the most updated version of this article at doi: 10.1074/jbc.RA117.000505

**Alerts:**
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts