

Filamin A-interacting protein (FILIP) regulates cortical cell migration out of the ventricular zone

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Precisely regulated radial migration out of the ventricular zone is essential for corticogenesis. Here, we identify a mechanism that can tether ventricular zone cells *in situ*. FILIP interacts with Filamin A, an indispensable actin-binding protein that is required for cell motility, and induces its degradation in COS-7 cells. Degradation of Filamin A is identified in the cortical ventricular zone, where *filip* mRNA is localized. Furthermore, most ventricular zone cells that overexpress FILIP fail to migrate in explants. These results demonstrate that FILIP functions through a Filamin A–F-actin axis to control the start of neocortical cell migration from the ventricular zone.

An unique cell migratory programme in neocortical development has recently been recognized: whereas some GABAergic neurons migrate into the neocortex¹ and lateral olfactory tract guidepost neurons migrate from the neocortex², radial migration of neurons from the ventricular zone within the neocortex towards the pial surface is essential and supplies most neocortical excitatory neurons^{3–6}. In this radially migrating scheme, post-mitotic neurons generated in the ventricular zone have to make at least two important decisions: when to start and where to stop migration to reach their destinations correctly and form the six well-ordered layers of the neocortex⁷. Since the extracellular protein Reelin was identified as a key molecule in the control of where to stop migration, the underlying mechanisms have been studied extensively^{8–15}. By contrast, the molecular basis of when to start migration is poorly understood. So far, it has been demonstrated that post-mitotic neurons are already destined to their layer fates in the ventricular zone before they start radial migration¹⁶ and that neurons of layers 2–6 are generated in an ‘inside–out’ fashion, in which those destined for layer 6 leave out of the ventricular zone first and those destined for layer 2 do so last^{7,17}. Therefore, ‘when to start migration’ is essential for the establishment of neocortical layers.

A clue to the starting mechanism comes from the observation that a mutation in Filamin A (also known as Filamin 1 and ABP-280)¹⁸ results in periventricular nodular heterotopia, a human neuronal migration disorder in which many post-mitotic neurons remain on the ventricular surface^{19,20}. Filamin A crosslinks F-actin into isotropic, orthogonal arrays and increases the viscosity and stiffness of the F-actin network^{18,21–24}. Filamin A is essential for radial cell migration, but its expression from the intermediate zone to the cortical plate, both in migratory and post-migratory neurons of the developing neocortex²⁰, suggests that other molecules are also involved in controlling the start of migration from the ventricular zone.

In this study, we identified FILIP through a search for genes in the ventricular zone that were expressed more abundantly when most post-mitotic neurons were just about to migrate than when many neurons had already left. FILIP negatively regulated the function of Filamin A, and *filip* mRNA was expressed in ventricular zone cells, but not in migratory or post-migratory cells. These findings describe how FILIP and Filamin A can regulate the start of neocortical cell migration from the ventricular zone.

Results

Identification of FILIPs. To identify the molecules responsible for regulating the start of radial cell migration during neocortical development, we used mRNA differential display to isolate genes expressed more abundantly in the neocortices of Wistar rats on embryonic day 11–12 (E11–E12) compared with E18–E20. At E12, post-mitotic neurons are just about to migrate out of the ventricular zone towards the pial surface. By E20, however, most of them have already left the ventricular zone, when neurogenesis is complete⁷. The 200 gene-fragments that were recognized as dominant at E12, but not at E18–E20, were sequenced to exclude overlap. Further selection was performed by *in situ* hybridization. Of 80 independent clones, one novel clone (designated later as *filip*) showed restricted expression in the ventricular zone of the neocortex (Fig. 1a). In addition to the nervous system, *filip* mRNA was also observed in muscular tissues, including the cardiovascular system. Two full-length FILIP cDNAs were eventually cloned, both of which include the region used as a probe for *in situ* hybridization selection. The two clones differed only in their 5′ termini. Their deduced amino acid sequences indicated that S-FILIP (short-form FILIP) lacks the amino-terminal 247 residues of L-FILIP (long-form FILIP), (Fig. 1b). These two FILIPs are intracellular proteins, because neither a signal sequence nor a transmembrane region was found in their hydrophobicity profiles (data not shown). Four

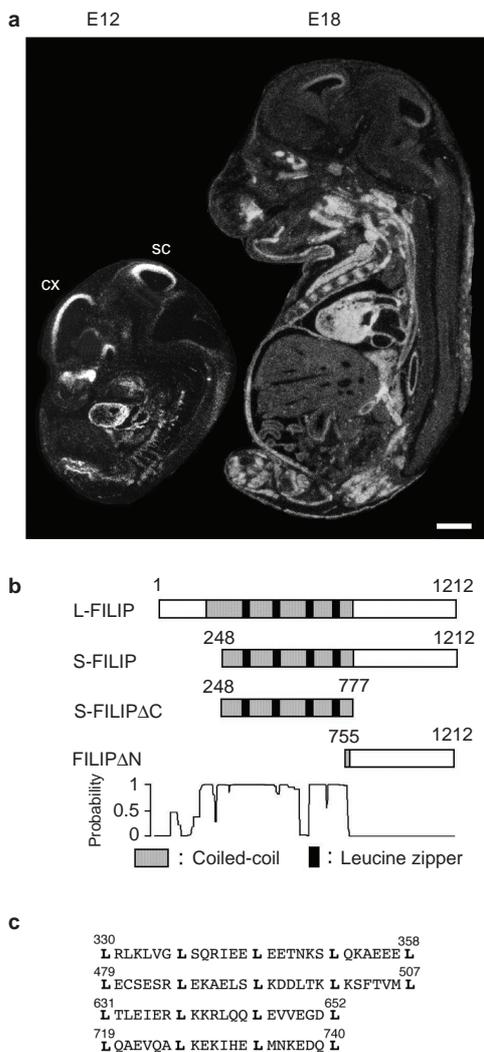


Figure 1 Expression and structure of FILIPs. **a**, *filip* gene expression was analysed in E12 and E18 rat sagittal sections by *in situ* hybridization. Positive signals were evident in the ventricular zone of the cerebral cortex (cx) and superior colliculus (sc) in the central nervous system in E12 (left). Signals in the ventricular zone were less intense in E18 (right), whereas robust signals were observed in the heart, great blood vessels, gastrointestinal tract and diaphragm, suggesting that the *filip* gene was expressed in cardiac, skeletal and smooth muscle. Scale bar, 1 mm. **b**, A schematic representation of FILIPs based on deduced amino acid sequences. Residues are numbered from the N-terminus of L-FILIP. S-FILIP lacks the 247 N-terminal residues of L-FILIP; the remainder of the structure of both FILIPs is identical. The grey areas represent the predicted coiled-coil regions, based on the coiled-coil conformation probability³¹ shown below (0–1 on the y axis; the probability was calculated using a window of 28 residues). In addition, 4 leucine zipper motifs were recognized within the N-terminal halves of the molecules (black rectangles); no similarity to any other reported proteins was noted in the C-terminal halves. **c**, Amino acid sequences of leucine zipper motifs found in FILIPs. Residues are numbered as in **b**.

leucine zipper motifs and a coiled-coil region could be recognized in their N-terminal halves (Fig. 1b,c).

Intracellular localization of FILIPs. To investigate the subcellular localization of S- and L-FILIP, FILIPs tagged with green fluorescent protein (GFP) at their carboxy termini (FILIPs–GFP) were expressed in COS-7 cells. Two patterns of FILIP localization were observed (Fig. 2a): first, GFP-tagged FILIPs localized typically

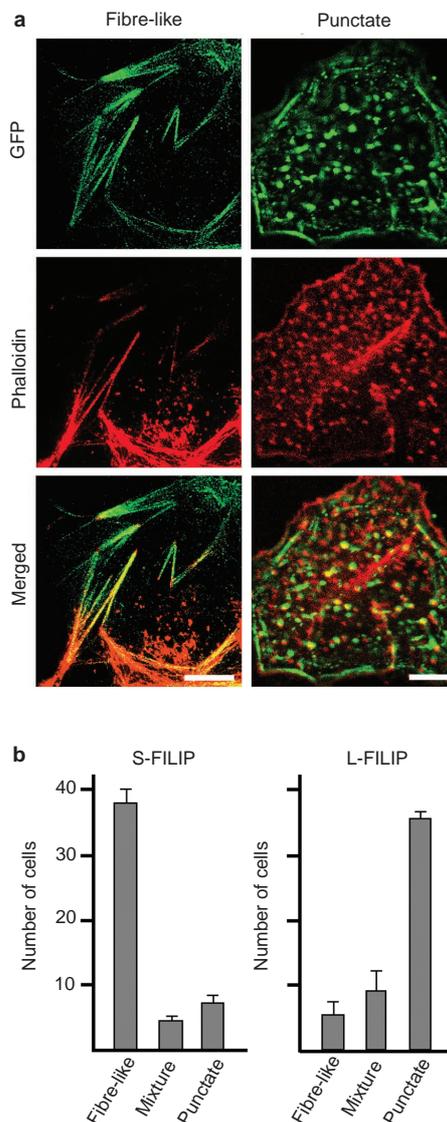


Figure 2 FILIPs are cytoskeleton-associated proteins. **a**, In COS-7 cells, GFP-tagged FILIPs showed fibre-like (left) and punctate (right) distributions. In cells with a fibre-like distribution, FILIPs colocalized with F-actin (visualized using rhodamine–phalloidin), whereas in cells with a punctate distribution, FILIPs did not colocalize with F-actin (middle and bottom). Scale bars, 10 μm. **b**, The numbers of cells with each GFP-tagged FILIP distribution pattern were counted among a randomly selected pool of 50 GFP-tagged S- or L-FILIP-expressing cells. Cells showing both patterns were counted as ‘mixture’. Results are shown as mean values (s.e.m. from four independent experiments). The fibre-like pattern was typically observed in S-FILIP-expressing cells, whereas the punctate pattern was observed in L-FILIP-expressing cells. **c**, Although the GFP-tagged C-terminal half of S-FILIP (FILIPΔC–GFP, see Fig. 1b), which has no known actin-binding domain, had a fibre-like pattern in COS-7 cells (middle), the GFP-tagged N-terminal half did not (left). This suggests that FILIPΔN is both necessary and sufficient to induce colocalization with F-actin. Scale bar, 20 μm.

along actin stress fibres, except at their ends, suggesting colocalization with F-actin (fibre-like); second, a punctate distribution in the cytoplasm was observed, which was distinct from that of F-actin (punctate). Some cells had a mixture of both patterns. These patterns were dependent largely on the species of FILIP examined (Fig. 2b). S-FILIP typically colocalized with F-actin, although no known actin-binding motif was found in its sequence. Its C-terminal half (FILIP Δ N), which was common to both FILIPs, was both necessary and sufficient for colocalization with F-actin (Fig. 2c and data not shown). By contrast, little L-FILIP colocalized with F-actin, and instead had a punctate distribution in the cytoplasm of most cells. In addition, actin stress fibres were rarely found in COS-7 cells expressing L-FILIP.

FILIP interacts with Filamin A. The unique colocalization of S-FILIP with F-actin prompted us to search for molecules that may provide a link between these molecules. We used a yeast two-hybrid screen with the C-terminal half of S-FILIP and mouse E11 whole-embryo library as bait and prey, respectively. In this way, we identified a clone that encodes Filamin A. This clone corresponds to a middle part of Filamin A, spanning the C-terminal end of β -sheet repeat 15 to the middle of repeat 18, and therefore includes the hinge 1 region that can be cleaved by the protease calpain²². An interaction of S-FILIP, both the full-length sequence and the C-terminal half, with Filamin A was confirmed by immunoprecipitation (Fig. 3a,b). Colocalization of S-FILIP-GFP with endogenous Filamin A was further corroborated by immunocytochemical analyses in COS-7 cells. Most S-FILIP signals overlapped with Filamin A (Fig. 3c, fibre-like). Thus, it is likely that S-FILIP colocalizes with F-actin through an interaction between the C-terminal half of FILIP and Filamin A. As the C-terminal half of L-FILIP is identical to that of S-FILIP, it is assumed that L-FILIP will also associate with Filamin A. This was confirmed by immunoprecipitation and immunocytochemical analyses (Fig. 3). In L-FILIP-expressing cells, approximate 50% of the punctate signals of FILIP colocalized with those of Filamin A (Fig. 3c). Both the fibre-like and punctate patterns of FILIP signals colocalized, at least in part, with endogenous Filamin A.

FILIPs suppress cell motility *in vitro*. As Filamin A is essential for cell motility in various cells²⁵, it is likely that FILIPs regulate cell migration through Filamin A. Thus, we introduced exogenous FILIPs into COS-7 cells, which possess endogenous Filamin A but not FILIPs. Under low cell density conditions, in which cells can move freely, those expressing FILIPs-GFP had reduced motility when compared with control cells (Fig. 4a,b). We also performed a wound-healing assay to determine the significance of FILIPs in lamellipodium formation; in this assay, quiescent cells in an over-confluent state usually develop lamellipodia (sheet-like processes) in response to the removal of neighbouring cells. In contrast to cells without FILIPs, most cells expressing FILIPs-GFP at the wound edge did not form lamellipodia (Fig. 4c); 68% of cells expressing GFP alone (control) at the wound edge formed lamellipodia, whereas only 28% with S-FILIP-GFP and 4% of cells with L-FILIP-GFP did so ($n=50$ for each experiment). These results indicate that FILIPs suppress cell motility and the formation of lamellipodia, suggesting an inhibitory role for FILIPs in Filamin A function.

FILIPs induce degradation of Filamin A. We further examined the molecular basis for the inhibitory effect(s) of FILIPs on Filamin A through co-expression of recombinant FILIPs and recombinant Filamin A in COS-7 cells. In these experiments, we used a single expression vector with dual promoters (Fig. 5a). HA-tagged Filamin A (HA-Filamin A) and GFP were translated from a single mRNA in the same cell, with or without expression of FILIPs. The amount of HA-Filamin A expressed relative to GFP was 4.7 in the absence of FILIPs and 1.8 in the presence of S-FILIP. However, no obvious band for HA-Filamin A was recognized in the presence of L-FILIP, whereas expression of GFP was clearly observed, demonstrating that there is little HA-Filamin A protein in L-FILIP-expressing cells, even with substantial amounts of HA-Filamin A

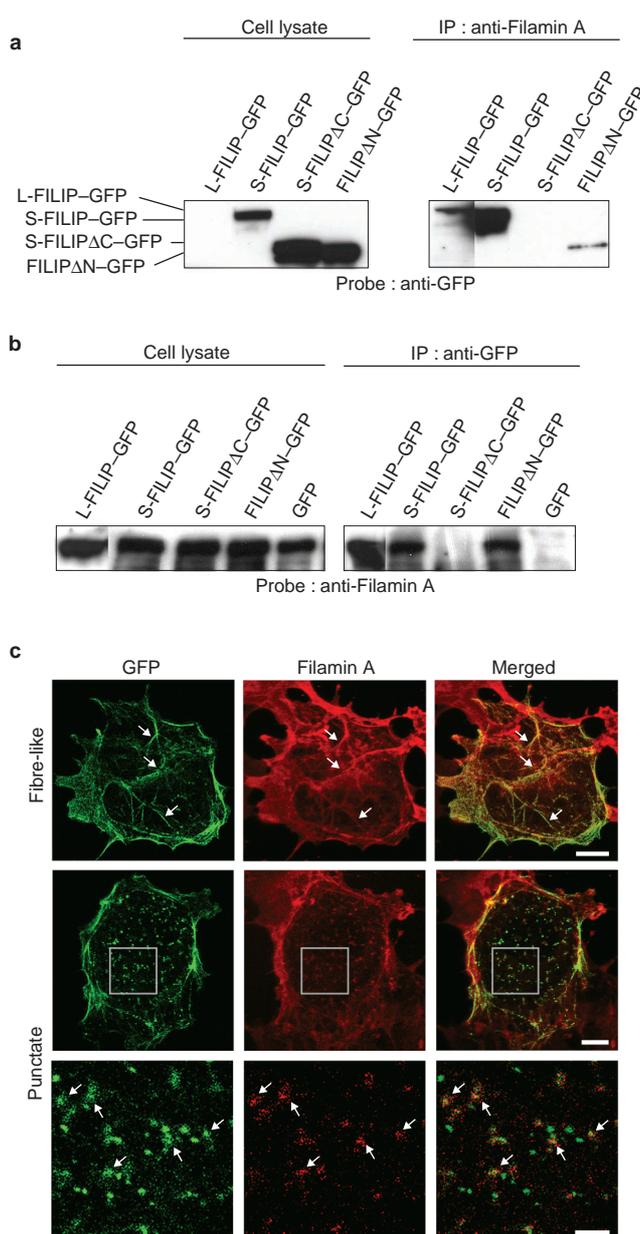


Figure 3 FILIPs interact with an actin-binding protein, Filamin A. a, b, Lysates from COS-7 cells expressing either L-FILIP-GFP, S-FILIP-GFP, S-FILIP Δ C-GFP, FILIP Δ N-GFP or GFP only were immunoprecipitated with either anti-Filamin A (a) or anti-GFP (b) antibodies and then immunoblotted with anti-GFP or anti-Filamin A antibodies, respectively. 1% of each cell lysate was used for the cell lysate lanes. The remainder of the sample was processed for immunoprecipitation. Both L- and S-FILIP-GFP and FILIP Δ N-GFP were co-immunoprecipitated with endogenous Filamin A. c, COS-7 cells expressing S- or L-FILIP-GFP were analysed by immunocytochemistry using an anti-Filamin A antibody. Both fibre-like FILIP-GFP (top; typically S-FILIP-GFP) and punctate FILIP-GFP (middle and bottom; typically L-FILIP-GFP) colocalized, at least in part, with endogenous Filamin A, supporting the results shown in a and b. Higher magnification views of the square in the middle panels are also shown (bottom). Arrows indicate colocalization of FILIPs-GFP and Filamin A. Scale bars, 10 μ m (top and middle), 3 μ m (bottom).

and GFP mRNA. This was consistent with weak immunoreactivity for Filamin A in COS-7 cells expressing L-FILIP-GFP (Fig. 5b). By contrast, HA-Filamin A protein was effectively accumulated in the presence of calpeptin, an inhibitor of the calcium-dependent

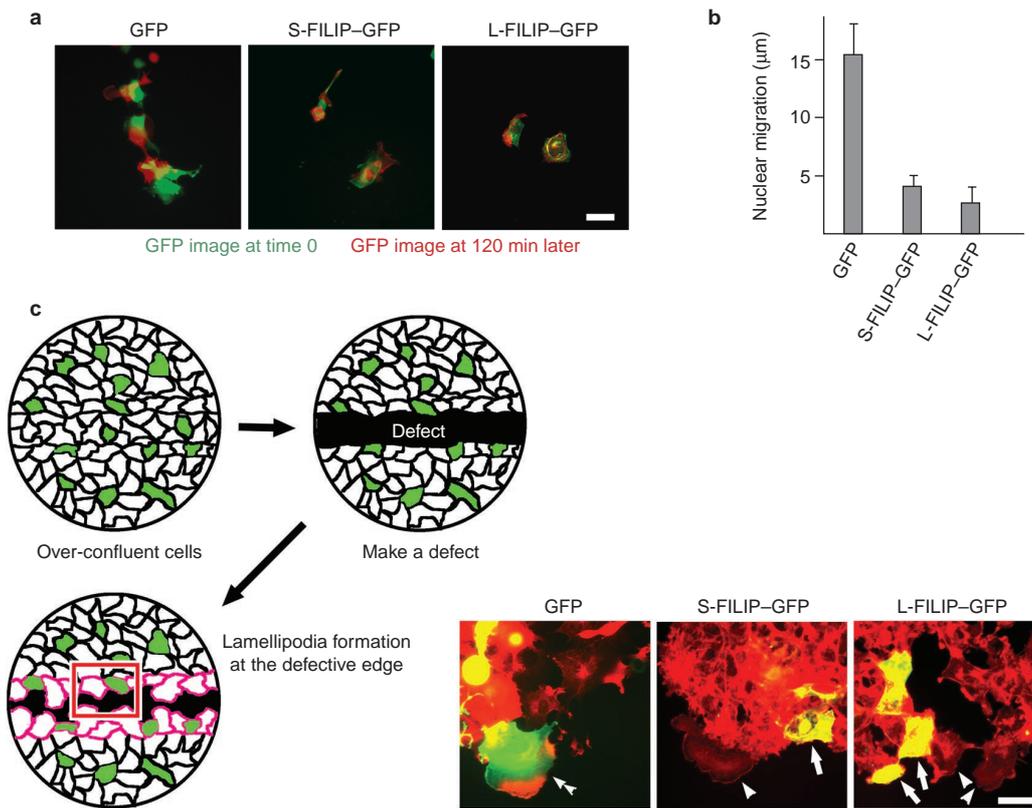


Figure 4 FILIPs suppress cell motility and the formation of lamellipodia in COS-7 cells. **a**, COS-7 cells expressing GFP (left), S-FILIP-GFP (middle) or L-FILIP-GFP (right) were cultured under low cell density conditions. GFP images (green) were acquired at an interval of 120 min and merged together after the colour of the later images had been converted to red. Cells did not exhibit active movement in the presence of FILIPs. Scale bar, 50 μm. **b**, To quantify cell motility in **a**, the migrated distances (mean with s.e.m.) of each nucleus during the 120-min interval was measured for each group ($n = 18$ for GFP alone, $n = 20$ for S-FILIP-GFP and $n = 19$ for L-FILIP-GFP). **c**, Wound healing assay. After making a

defect among over-confluent COS-7 cells, the number of cells with lamellipodia in 50 randomly selected cells at the wound edge showing green GFP signals (FILIPs-GFP or GFP alone) was counted. The red square corresponds to the region where images were taken. Both S- and L-FILIP-GFP-expressing cells at the wound edge (middle and right images) had a reduced tendency to develop lamellipodia (arrows), compared with cells expressing GFP alone (left image, double arrowhead). Note that neighbouring cells without FILIPs (arrowheads) responded normally. Cells were stained for actin with rhodamine-phalloidin (red). Scale bar, 50 μm.

protease, calpain (Fig. 5a). These results suggest that degradation of Filamin A is activated by calpain or equivalent calpeptin-sensitive protease(s) in the presence of FILIPs. This idea is further supported by the observation that degradation of Filamin A was suppressed by the intracellular calcium-chelating reagent, 1,2-bis (2-aminophenoxy)-ethane-N,N,N',N'-tetra acetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), but was resistant to the proteasome inhibitor, MG-132 (Fig. 5a). Thus, Filamin A protein that associates with FILIPs is induced to degrade. Therefore, this is likely to be one possible reason for the weak immunoreactivity for Filamin A that colocalized with FILIPs, especially those with the punctate pattern (Fig. 3c). Next, we examined whether the degradation of Filamin A could be affected by its other binding partner, F-actin. HA-tagged Filamin A lacking the actin-binding domain (HA-Filamin A-ΔABD; the actin-binding domain of Filamin A corresponds to the N-terminal 274 residues²²) was used instead of HA-Filamin A. We found that the relative amount of HA-Filamin A-ΔABD to GFP was not reduced in the presence of L-FILIP, compared with that in the absence of L-FILIP (Fig. 5a). This suggests that L-FILIP preferentially induces degradation of F-actin-associated Filamin A. As Filamin A exerts its effects in F-actin dynamics¹⁸, this seems to be a reasonable feature of a Filamin A-regulating protein.

FILIPs suppress radial cell migration. Because FILIPs introduced into COS-7 cells exerted inhibitory effects on cell motility, as well as

inducing degradation of Filamin A, and mutated *filamin A* resulted in human cortical malformation in which affected post-mitotic cells remained in the ventricular zone^{19,20}, it is likely that FILIPs are pivotal in the control of cell migration in the developing neocortex. To examine the function of FILIPs in neuronal migration *in vivo*, we introduced FILIP cDNAs into the E18 rat neocortex by electroporation, so that a fraction of ventricular zone cells that were presumably destined to be upper layer neurons overexpressed FILIPs. After gene transfer by electroporation from the ventricular surface, transfected cortices were dissected out and cultured for 4 days. In preparations that had been transfected with control GFP, many cells labelled in the ventricular zone migrated out towards the pial surface (Fig. 6a,b; top). These migrating cells were spindle-shaped, with leading and trailing processes orientated radially, and identified as neurons²⁶. In contrast, cells expressing S- or L-FILIP-GFP were quite different in shape and motility from those labelled with GFP only. They were round, devoid of long and radially orientated processes, and hardly migrated (Fig. 6a,b; middle and bottom). The effects of FILIPs in ventricular zone cells were consistent with those in COS-7 cells. Fewer cells expressed L-FILIP-GFP, compared with GFP or S-FILIP-GFP. This was probably caused by the low efficiency of transfection or translation, because the number of cells expressing L-FILIP-GFP did not change significantly through the period of culture (data not shown).

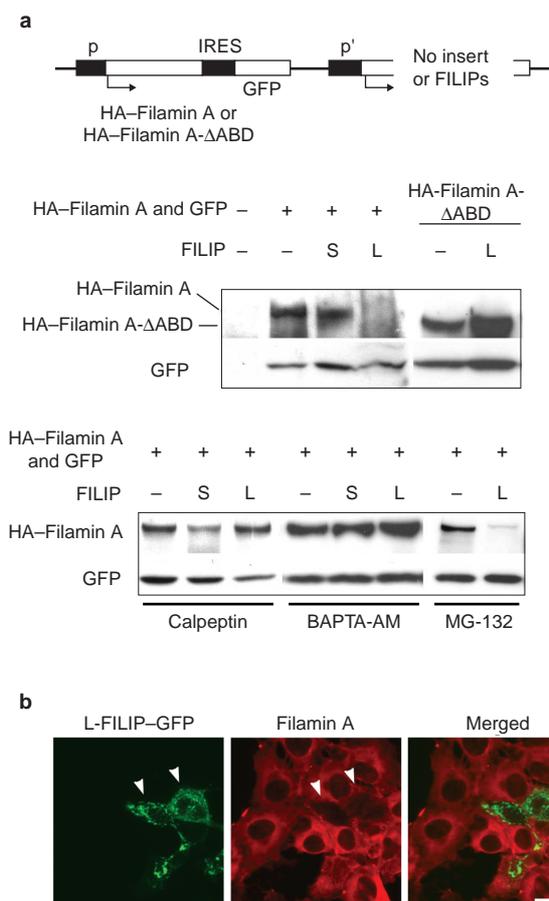


Figure 5 FILIPs induce degradation of Filamin A. **a**, Recombinant Filamin A and FILIPs were co-expressed in COS-7 cells. The upper drawing shows the structure of the vector used. An internal ribosome entry site (IRES) sequence was inserted between the HA-tagged Filamin A (HA-Filamin A) and GFP cDNAs. They were driven by a CMV promoter (p), whereas expression of FILIPs was driven by the EF-1 α promoter (p'). HA-Filamin A and GFP were translated from the same mRNA in the same cell. The relative amounts of HA-Filamin A expressed in COS-7 cells in the absence (-) or presence of either S-FILIP (S) or L-FILIP (L) could be estimated by comparison with expression of GFP, by immunoblotting with anti-HA and anti-GFP antibodies, respectively. Approximately 48 h after transfection, the relative amounts of HA-Filamin A, but not those of Filamin A- Δ ABD, were decreased in the presence of FILIPs, especially L-FILIP (middle). These effects were diminished in the presence of calpeptin (50 μ M) or BAPTA-AM (20 μ M), suggesting that FILIPs induce the degradation of Filamin A (bottom). MG-132 (10 μ M) had no obvious effect. **b**, Typically, in COS-7 cells expressing L-FILIP-GFP (arrowheads, green), the amount of endogenous Filamin A (red) was markedly reduced when compared with neighbouring cells without FILIP. Scale bar, 25 μ m.

Degradation of Filamin A in the ventricular zone. FILIPs, especially L-FILIP, were expressed dominantly during the late embryonic period, when corticogenesis is in progress (Fig. 7a). Although expression of Filamin A protein has been detected predominantly in the migrating and post-migratory neurons of the intermediate zone and cortical plate of human embryonic brain²⁰, our *in situ* hybridization study demonstrated robust expression of *filamin A* mRNA in the ventricular zone, as well as in the cortical plate (Fig. 7c). Because *filips* mRNAs were expressed in the ventricular zone, it is likely that FILIPs interact with Filamin A and induce its degradation there. Indeed, our immunoprecipitation experiment demonstrated that endogenous FILIP (mainly L-FILIP) interacted with endogenous

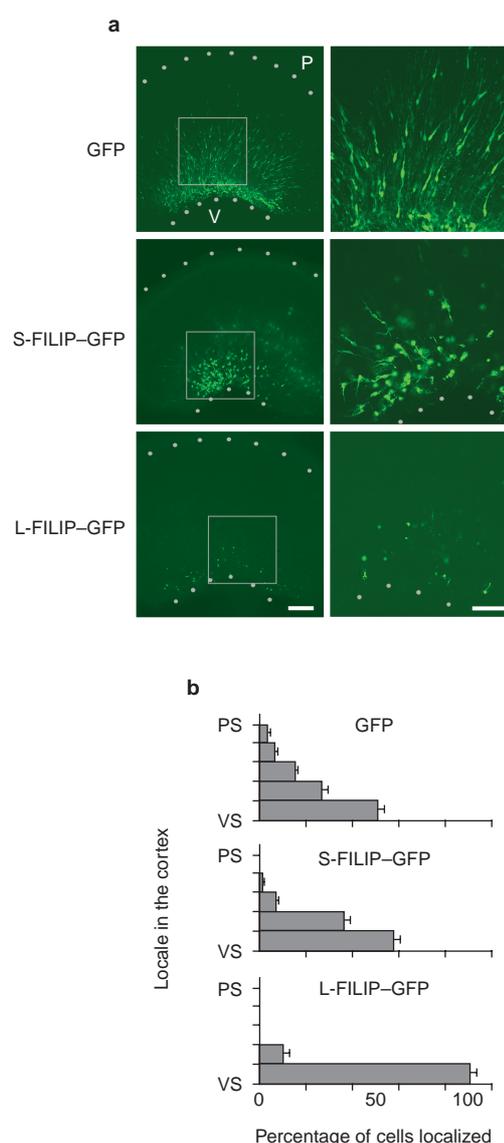


Figure 6 FILIPs suppress radial cell migration in neocortical explants. **a**, cDNAs encoding GFP (top), S-FILIP-GFP (middle) or L-FILIP-GFP (bottom) were transfected into the ventricular zone of E18 rat cortex by electroporation. Dissected cortices were maintained as explants for 4 days. Radially migrating post-mitotic neurons were labelled with GFP, whereas most cells expressing S- or L-FILIP-GFP were round and remained close to the ventricular zone. The right column shows higher magnification views of each square in the left column. Dots indicate the edges of the explants. P, pial surface; V, lateral ventricle. Scale bars, 200 μ m (left column), 100 μ m (right column). **b**, Quantitative analyses of **a**. The neocortex was subdivided evenly into five areas from the ventricular side (VS) to the pial surface (PS). Percentages of GFP-labelled cells (cell bodies) in each area were counted after 4 days in culture (three explants for GFP and S-FILIP-GFP and five explants for L-FILIP-GFP). Values are means with s.e.m.

Filamin A in the E12 rat cortex (Fig. 7b). Furthermore, a large number of cells in the ventricular zone of E16 rats exhibited less immunoreactivity for Filamin than those in the intermediate zone and cortical plate (Fig. 7d). These results support the hypothesis that FILIP induces the degradation of Filamin A in the ventricular zone.

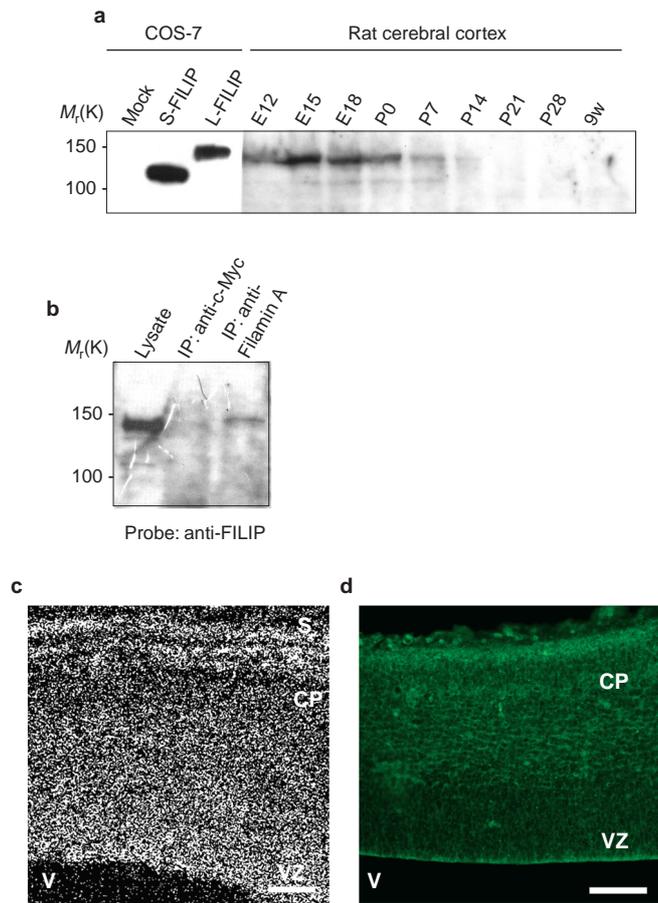


Figure 7 L-FILIP interacts dominantly with Filamin A in the developing neocortex. **a**, Ontogenetic expression profiles of L- and S-FILIP in developing rat neocortex were analysed by immunoblotting. L-FILIP was more abundant than S-FILIP. The anti-FILIP antibody recognized both S- and L-FILIP expressed in COS-7 cells with high specificity. **b**, L-FILIP was found in the lysate of E12 rat neocortices, whereas S-FILIP was barely detectable (left lane). L-FILIP was co-immunoprecipitated from the same lysate with the anti-Filamin A antibody (right lane), whereas the anti-c-Myc antibody did not generate any positive signal (middle lane). **c**, *In situ* hybridization using E16 rat neocortex showed that expression of *filamin A* gene was observed throughout the developing cortex, with particularly strong expression detected in the ventricular zone (VZ). CP, cortical plate; S, skull; V, lateral ventricle. Scale bar, 100 μ m. **d**, Immunohistochemistry using E16 rat neocortex showed that Filamin-like immunoreactivity in the ventricular zone (VZ) was less than that observed in the cortical plate (CP) and the intermediate zone, although the ventricular zone cells strongly expressed the *filamin A* gene (see **c**). Scale bar, 100 μ m.

Discussion

Because ventricular zone cells in the neocortex express *filip* mRNA, and Filamin A is essential for radial cell migration out of the ventricular zone²⁰, it is highly likely that FILIP regulates the start of radial migration from the ventricular zone; ventricular zone cells are tethered *in situ* as long as they express FILIP, through a loss of Filamin A, whereas they are prepared to leave the ventricular zone when FILIP activity decreases. This represents a unique molecular basis for regulating the migration start, in which FILIP generates an ‘intracellular environment’ against migration during the pre-migratory stage. The start of radial migration actually succeeds neurogenesis within minutes in the ventricular zone^{5,6}. Therefore, it is likely that a rapid increase of Filamin A levels, as well as quick inactivation of FILIP activity, is required to ensure punctual departure for post-mitotic

neurons. We demonstrated that FILIP preferentially degrades F-actin-associated Filamin A, but not F-actin-free Filamin A, in a calcium-dependent manner (Fig. 5a). This calcium dependency fulfils an apparent requisite for FILIP activity in the ventricular zone; FILIP activity can be quickly inactivated whenever necessary. In addition, a pool of F-actin-free Filamin A protein, which escapes FILIP-induced degradation, would function as a reservoir, allowing a rapid response to requirements for Filamin A.

The fibre-like distribution of FILIPs was observed in a large fraction of cells (fibre-like cells) expressing S-FILIP, whereas a punctate distribution was observed in those cells expressing L-FILIP (Fig. 2b). Our observations that L-FILIP was more active than S-FILIP in the degradation of Filamin A (Fig. 5), as well as in suppressing the formation of lamellipodia and inhibiting cell motility (Fig. 4), suggested that Filamin A was degraded in cells with the punctate pattern. Moreover, the actin organization is severely disturbed in these cells (Fig. 2a). It is likely that this disorganization is caused by the degradation of Filamin A *per se*, as Filamin A is indispensable for actin organization¹⁸. On the other hand, most S-FILIP expressing cells were fibre-like cells (Fig. 2b). As the distribution of F-actin was not apparently disorganized in such fibre-like cells (Fig. 2a), we suggest that Filamin A was not sufficiently degraded to induce impairment of the whole F-actin network, presumably because of the weak ability of S-FILIP to induce Filamin A degradation (Fig. 5a). However, despite the fact that approximately 75% of S-FILIP-expressing cells are fibre-like, S-FILIP-expressing COS-7 cells migrated only approximately 30% the distance of control cells with a small s.e.m. (Fig. 4), indicating that almost all S-FILIP-expressing cells have reduced cell motility. Therefore, in most fibre-like cells it is probable that some Filamin A was degraded and the actin organization was distorted to some extent. It seems that precise coordination of Filamin A and F-actin is fundamental for active cell movement. In addition, it is apparent that Filamin A and actin organization are involved in radial migration in the neocortex, based on the fact that overexpression of S- or L-FILIP inhibits radial migration of neocortical cells. As L-FILIP is more potent than S-FILIP in suppressing the formation of lamellipodia and inhibiting cell motility, and L-FILIP was more dominant during corticogenesis (Fig. 7a), it is possible that L-FILIP is more important than S-FILIP in the developing neocortex.

filip mRNA is widely expressed in embryos (Fig. 1). Expression of *filip* mRNA in the ventricular zone of the superior colliculus suggests that FILIP also functions in this region, as the organized layer formation is one typical feature of the superior colliculus, just as in the neocortex²⁷. Also, robust expression of *filip* mRNA in the muscular tissue is notable (Fig. 1a). Because Filamin C (also referred as γ -Filamin or ABP-L) is the dominant isoform in muscular tissue and has a similar FILIP binding region to Filamin A^{18,28}, it is possible that FILIP interacts with and modifies the function of Filamin C in most muscular tissues. Filamins are abundant cellular proteins with pivotal functions in various cells; in addition to their roles in F-actin organization and cell motility, some Filamins are critical in signalling functions¹⁸. Many factors bind to Filamin and modulate its activities¹⁸. Unlike other Filamin-binding proteins, FILIP induces degradation of Filamin. This characteristic is unique among Filamin-binding partners. □

Methods

Animals

Wistar rats were purchased from Kears (Osaka, Japan) and SLC (Hamamatsu, Japan). They were housed at a constant temperature and humidity and provided with food and water *ad libitum*. Embryonic day 0 (E0) is defined as the day of confirmation of the vaginal plug and the day of birth is designated as P0 (postnatal day 0). All animals were deeply anaesthetized by hypothermia (for P0 to P7) or by intraperitoneal injection of sodium pentobarbital (40 mg kg⁻¹ for P14 to adult, including dams) before experiments. All experiments were conducted in accordance with the guidelines for use of laboratory animals (Fukui Medical University, Osaka City University and National Institute for Basic Biology).

Isolation of FILIP cDNAs and *in situ* hybridization

Procedures for mRNA differential display, *in situ* hybridization and screening of the rat cDNA library were performed essentially as previously described²⁹. We constructed a Wistar rat E11 forebrain cDNA

library and used it for our initial screening for S-FILIP. A Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) was used to obtain the full-length S-FILIP cDNA. Genetic information from the DNA Databank of Japan database was also utilized in part to isolate full-length L-FILIP cDNA.

Cell culture, DNA transfection, immunocytochemistry and analyses for cell motility

COS-7 cells were maintained in DMEM containing 10% foetal bovine serum (FBS) at 37 °C in 5% CO₂. Mammalian expression vectors (pEGFP-N1 (Clontech), pCAGGS and pBudCE4 (Invitrogen, Carlsbad, CA)) containing either various FILIPs, Filamin A and/or GFP cDNAs were transfected using TransFast transfection reagent (Promega, Madison, WI) or PolyFect transfection reagent (Qiagen, Hilden, Germany). Reagents were used at following concentrations: calpeptin (Tocris Cookson Ltd., Bristol, UK), 50 μM; MG-132 (Biomol Research Laboratories Inc., Plymouth Meeting, PA), 10 μM; BAPTA-AM (Sigma, St Louis, MO), 20 μM.

For F-actin staining, cells were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (PB) at pH 7.4 for 10 min, permeabilized in 0.1% Triton X-100/PBS for 3 min, and stained with rhodamine-phalloidin (dilution 1:40; Molecular Probes, Eugene, OR). For immunocytochemical staining against Filamin A, cells were fixed and permeabilized as described above, blocked in 10% normal goat serum/PBS for 20 min and incubated with an anti-Filamin A antibody (dilution 1:200; Chemicon, Temecula, CA) and then with an anti-mouse Ig-Cy3 antibody (dilution 1:400; Amersham Biosciences, Piscataway, NJ). They were imaged on either an Olympus IX-70 microscope equipped with a digital cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan), or an Olympus GB-200 laser-scanning confocal microscope.

For analysis of cell motility under low cell density condition, COS-7 cells were transfected with the expression vectors on the day after plating (approximately 1 × 10⁴ cells per 1.88 cm² area). After 36–48 h, cells were cultured in the same medium described above on an IX-70 microscope equipped with an IX-IBC culturing apparatus (Olympus, Tokyo, Japan) and imaged twice at an interval of 120 min. These two images were merged after the later ones had been converted to red colour. Nuclear migration during the interval was quantified using phase-contrast images.

For the wound-healing assay, over-confluent COS-7 cells were transfected with the expression vectors. After 36–48 h, defects were made among the cells (see Fig. 4c) and then cultured in the medium described above for a further 3 h. After fixing and staining with rhodamine-phalloidin, cells at the defect edge containing either FILIPs-GFP or GFP alone were observed, to determine whether or not they had lamellipodia.

Two-hybrid screen

We used the Matchmaker Two-Hybrid system (Clontech). The yeast strain PJ69-2A was transformed with the pAS2-1 plasmid vector containing a cDNA encoding the common C-terminal region of FILIPs (residues 755–1212 of the deduced amino acid sequence of L-FILIP), and mated with E11 mouse brain pre-transformed Matchmaker library (Clontech). Over 8 × 10⁶ clones were screened and 17 clones were finally selected, based on their triple selection markers.

Immunoprecipitation

Protein samples from COS-7 cells or rat neocortices (solubilized in 20 mM Tris-HCl at pH 7.5, 150 mM sodium chloride, 1000 U ml⁻¹ DNase I, 1% NP-40, 1 mM phenyl-methanesulfonyl fluoride, 5 μg ml⁻¹ aprotinin, 1.5 μM pepstatin A and 2 μM leupeptin) were immunoprecipitated using either anti-Filamin A (Chemicon), anti-GFP (Clontech) or anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The precipitated proteins were then analysed by immunoblotting.

Neocortical slice culture and DNA transfection

The methods of neocortical slice culture and DNA transfection have been described elsewhere²⁶. In brief, plasmid DNA was injected into the lateral ventricle of E18 rat brain, then introduced into ventricular zone cells by delivering electrical pulses with a square-pulse electroporator. The brain was cut coronally into 200-μm sections with a microtome, and the dorsal portion of the cortex was dissected out and cultured on a collagen-coated membrane (Transwell-COL, Costar-Corning, Corning, NY) in DMEM/F12 medium containing 10% FBS and N2 supplement. Sections were fixed with 4% paraformaldehyde/0.1 M PB at pH 7.4 and imaged on a Zeiss LSM510 laser-scanning confocal microscope (Zeiss Instruments, Göttingen, Germany). The migration of each cell with GFP or FILIPs-GFP was quantified on the basis of cortical locales, where its cell body was found at day 4 of culture.

Anti-FILIP antibody

A polyclonal anti-FILIP antibody was prepared, as described elsewhere³⁰. Briefly, a rabbit was immunized with a synthetic peptide corresponding to residues 1139–1156 of the deduced amino acid sequence of L-FILIP, which is also contained in S-FILIP.

Immunohistochemistry

Frozen sections prepared from E16 rat cerebral cortices were fixed in Zamboni's solution (0.1 M PB at pH 7.4, 2% paraformaldehyde and 0.21% picric acid), air-dried and permeabilized with 0.2% Triton X-100 and 0.5% bovine serum albumin/PBS for 30 min. They were then incubated with an anti-Filamin antibody (1:40; Sigma) and anti-goat IgG-fluorescein (dilution 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA).

Accession numbers

Rat S-FILIP and L-FILIP cDNA sequences are deposited in GenBank under accession numbers D87257 and AB055759, respectively.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.