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Involvement of Filamin A and Filamin A-interacting protein (FILIP) in controlling the start and cell shape of radially migrating cortical neurons

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Abstract

Precisely regulated radial cell migration out of the ventricular zone is essential for corticogenesis. However, molecular mechanisms controlling the start of migration and the dynamics of migrating cell shape remain elusive. Here, we show novel mechanisms that can tether ventricular zone cells and control migrating cell shape. The novel protein Filamin A-interacting protein (FILIP) interacts with Filamin A, an indispensable actin-binding protein for cell motility, and induces its degradation in COS-7 cells. Degradation of Filamin A is indicated in the cortical ventricular zone where *FILIP* mRNA localizes. Furthermore, most ventricular zone cells that overexpress FILIP fail to migrate in explants. These results indicate that FILIP acts through a Filamin A–F-actin axis to control the start of neocortical cell migration from the ventricular zone. Filamin A also determines the shape of migrating neocortical neurons, which show global morphological changes and complicated behavior during that migration. Dysfunction of Filamin A, caused by a mutant Filamin A expression, prevents cells from acquiring consistent polarity toward specific direction and decreases motility in the subventricular and intermediate zones. In contrast, Filamin A overexpression, achieved by a short interfering RNA for *FILIP*, promotes the development and maintenance of a bipolar shape also in the subventricular and intermediate zones. These results suggest that the amount of Filamin A helps migrating neurons determine their mode of migration, multipolar or bipolar, prior to entering the cortical plate and that FILIP is responsible, at least in part, for the Filamin A content of migrating neurons.

Key words: actin, cortex, cytoskeleton, development, migration, multipolar migration, neuroepithelium, periventricular heterotopia, polarity, ventricular zone.

Introduction

A unique cell migratory program used in neocortical development has recently been recognized: whereas some GABAergic neurons tangentially immigrate into the cerebral neocortex (Anderson *et al*., 1997), radial migration of neurons from the neocortical ventricular zone towards the pial surface supplies most neocortical excitatory neurons (Rakic, 1972; Miyata *et al*., 2001; Nadarajah *et al*., 2001; Noctor *et al*., 2001). 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, in order to reach their destinations correctly and form six well-ordered layers, which is the specific property of the neocortex (Bayer & Altman, 1991). Since the extracellular protein Reelin was identified as a key molecule for the latter decision, the underlying mechanisms of radial cell migration have been studied extensively (D'Arcangelo *et al*., 1995, 1999; Howell *et al*., 1997; Sheldon *et al*., 1997; Hiesberger *et al*., 1999; Senzaki *et al*., 1999; Trommsdorff *et al*., 1999; Dulabon *et al*., 2000). In contrast, the molecular basis for the former decision has been poorly elucidated. So far, it has been demonstrated that post-mitotic neurons are already destined to their layer fates in the ventricular zone prior to their start of radial migration (McConnell & Kaznowski, 1991) and that neurons of layers 2–6 are generated in an 'inside-out' fashion, in which those destined to layer 6 leave the ventricular zone first and those destined to layer 2 leave the ventricular zone last (Luskin & Shatz, 1985; Bayer & Altman, 1991). Therefore, 'when to start migration' is essential for the establishment of neocortical layers.

Furthermore, after leaving the ventricular zone, radially traveling neurons, from the ventricular zone to the cortical plate, exhibit varying morphology and

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motility (Rakic, 1972; Miyata *et al*., 2001; Nadarajah *et al*., 2001). It has been observed that abundant multipolar cells in the subventricular and intermediate zones (SVZ/IZ) show slow, irregular movement, together with dynamic extension and retraction of their multiple neurites (Tabata & Nakajima, 2003). Noctor *et al*. (2004) have described four distinct phases that migrating neurons undergo before entering the cortical plate, during which they halt and reverse direction. These observations suggest that radial migration through the SVZ/IZ is not simply a process to deliver neurons to the cortical plate, but has unexpected significance in regulating and/or coordinating corticogenesis through cellular interaction.

Therefore, how these morphological changes and complex behavior, as well as 'when to start migration', are regulated is an important issue for understanding formation of the neocortex.

Periventricular nodular heterotopia

Hereditary neocortical malformations that result from aberrant radial migration could provide important clues to understanding the molecular mechanisms that regulate corticogenesis. Several essential proteins, such as Lis1 and Doublecortin, have actually been identified through analyses of various malformations (Gupta *et al*., 2002). A clue for the starting mechanism is that the mutation in the actin-binding protein Filamin A (Filamin 1; ABP-280; Stossel *et al*., 2001) results in a human neuronal migration disorder, periventricular nodular heterotopia, in which many post-mitotic neurons remain lining the ventricular surface (Eksioglu *et al*., 1996; Fox *et al*., 1998). Filamin A crosslinks F-actin into isotropic, orthogonal arrays and increases the viscosity and stiffness of the F-actin network and is essential for cell motility (Brotschi *et al*., 1978; Gorlin *et al*., 1990; Cunningham, 1995; Glogauer *et al*., 1998; Stossel *et al*., 2001). Based on studies of periventricular nodular heterotopia, Filamin A is crucial for radial cell migration, but its expression in both migratory and post-migratory neurons in the developing neocortex, from the intermediate zone to the cortical plate (Fox *et al*., 1998) suggests that other molecules are also involved in the control over the start of migration from the ventricular zone.

Filamin A-interacting protein is a novel molecule expressed in the cortical ventricular zone

In order to elucidate the molecules responsible for regulating the start of radial cell migration during neocortical development, we searched for genes that are expressed more abundantly in the cortical ventricular zone of Wistar rats on embryonic day (E) 11–12 compared with E18–20 using mRNA differential display and *in situ* hybridization (Nagano *et al*., 2002). Post-mitotic neurons are just about to migrate out of the ventricular zone towards the pial surface on E12, whereas most of them have already left the ventricular zone at around E18–20, by which time neurogenesis is complete (Bayer & Altman, 1991). We finally identified a novel clone, which was later designated as *Filamin A-interacting protein* (*FILIP*), that showed restricted expression in the ventricular zone of the neocortex (Fig. 1A). Two full-length *FILIP* cDNAs were eventually cloned. Their deduced amino acid sequences indicated that the short-form FILIP (S-FILIP) lacks 247 residues of long-form FILIP (L-FILIP) at its N-terminus, whereas the rest of the sequences were identical (Fig. 1B). These two FILIP forms (S-FILIP and L-FILIP) are apparently intracellular proteins, because neither a signal sequence nor a transmembrane region was found in their hydrophobicity profiles (see Nagano *et al*., 2002).

Filamin A-interacting protein interacts with Filamin A

We used a yeast two-hybrid screen using the mouse E11 whole embryo library as prey to look for a binding partner of the apparent intracellular protein S-FILIP, then we identified a clone encoding Filamin A. Interaction of S-FILIP, both full-length and the C-terminal half, with Filamin A was confirmed by immunoprecipitation analyses (see Nagano *et al*., 2002). Colocalization of S-FILIP tagged with green fluorescent protein (S-FILIP–GFP) with endogenous Filamin A was further corroborated immunocytochemically in COS-7 cells. Most S-FILIP signals overlapped with Filamin A (Fig. 2). Thus, it is likely that S-FILIP colocalizes with Filamin A. Because 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 actually the case based on the immunoprecipitation analyses (see Nagano *et al*., 2002) and immunocytochemical results. In the case of L-FILIP-expressing cells, FILIP colocalized with endogenous Filamin A (Fig. 2). Because Filamin A is an actin-binding protein, colocalization of FILIP and F-actin was also confirmed (see Nagano *et al*., 2002). Accordingly, we designated these novel molecules as Filamin A-interacting proteins (FILIP).

Filamin A-interacting protein suppresses cell motility and induces degradation of Filamin A

Because Filamin A is essential for cell motility in various cells (Cunningham *et al*., 1992), it is probable

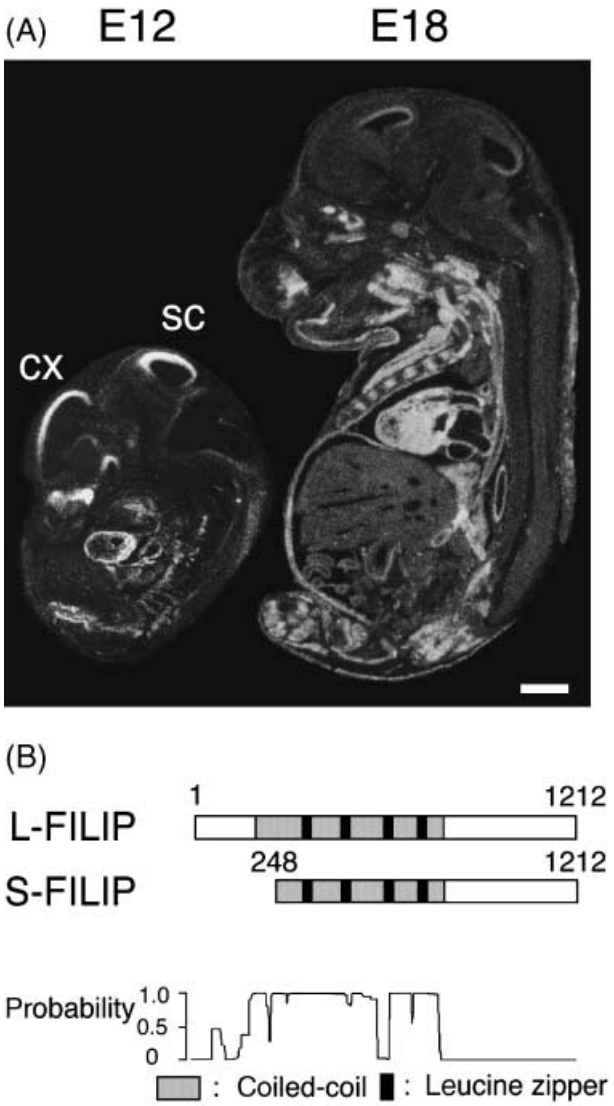

Figure 1. Expression and structure of Filamin A-interacting proteins (FILIPs). (A) *FILIP* gene expression was analyzed on embryonic day (E) 12 and E18 rat sagittal sections by *in situ* hybridization histochemistry. Positive signals were evident in the ventricular zone of the cerebral cortex (CX) and superior colliculus (SC) in the central nervous system on E12 (left). Signals in the ventricular zone were less intense on 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 muscles. Bar, 1 mm. (B) Schematic drawings of FILIPs based on the deduced amino acid sequences. Residues are numbered from the N-terminus of long-form FILIP (L-FILIP). Short-form (S-FILIP) lacks the 247 N-terminal residues of L-FILIP; the remainder of the structure of both FILIPs is identical. The hatched areas represent the predicted coiled-coil regions based on the coiled-coil conformation probability (Lupas *et al.*, 1991) shown below (from 0 to 1 on the *y*-axis; the probability was calculated using a window of 28 residues). In addition, four leucine zipper motifs were recognized within the N-terminal halves of the molecules (closed rectangles); no similarity to any other proteins reported so far was noted in the C-terminal halves. (Modified from Nagano *et al.*, 2002.)

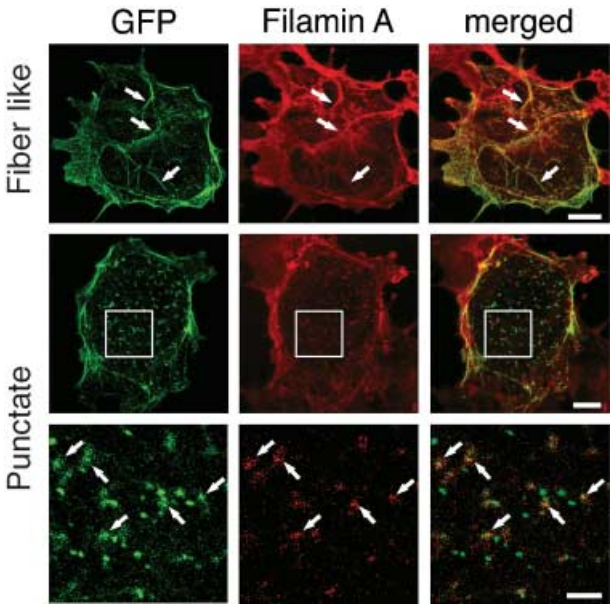

Figure 2. Filamin A-interacting proteins (FILIPs) interact with an actin-binding protein Filamin A. COS-7 cells expressing short-form (S-) or long-form (L-) FILIP–green fluorescent protein (GFP) were subjected to immunocytochemistry using anti-Filamin A antibody. Both fiber-like distribution of FILIP-GFP molecules (upper panels; typically S-FILIP-GFP) and punctate distribution (middle and lower panels; typically L-FILIP-GFP) colocalized, at least in part, with endogenous Filamin A. Lower panels show higher magnification views of the square in the middle panels. Arrows indicate signals for FILIP-GFP and Filamin A that colocalized with each other. Bars, 10 μm (upper and middle panels); 3 μm (lower panel). (Modified from Nagano *et al.*, 2002.)

that FILIPs regulate cell migration via Filamin A. Thus, we investigated whether or not FILIPs affect cell motility by introducing 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 without interfering with other cells, those cells expressing FILIPs–GFP showed reduced motility compared with control cells (Fig. 3A,B). This result indicates that FILIPs suppress cell motility, suggesting an inhibitory role of FILIPs on the function of Filamin A.

We then examined the molecular basis for the inhibitory effect(s) of FILIPs on Filamin A by simultaneous expression of recombinant FILIPs and recombinant Filamin A in the same COS-7 cells using a single expression vector with dual promoters (Fig. 4). In this experiment, HA-tagged Filamin A (HA-Filamin A) and GFP were translated from single mRNA in the same cell with the expression of FILIPs or not. 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 GFP expression was clearly observed,

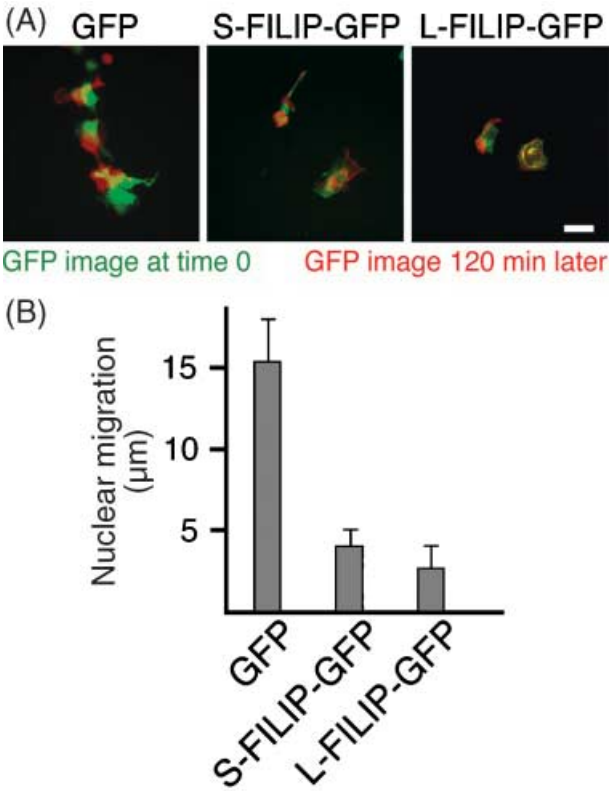

Figure 3. Filamin A-interacting proteins (FILIPs) suppress cell motility in COS-7 cells. (A) COS-7 cells expressing green fluorescent protein (GFP; left), short-form (S-) FILIP-GFP (middle) or long-form (L-) FILIP-GFP (right) were cultured under low cell density conditions and GFP images (green) were acquired at an interval of 120 min and were merged together after the color of the later images had been converted to red. Cells did not exhibit active movement in the presence of FILIPs. Bar, 50 μm. (B) To quantify cell motility in (A), migrated distances (mean ± SEM) of each cell nucleus during the 120 min interval was measured in each group (n = 18 for GFP alone; n = 20 for S-FILIP-GFP; n = 19 for L-FILIP-GFP). (Modified from Nagano *et al.*, 2002.)

demonstrating that there is little HA-Filamin A protein in L-FILIP-expressing cells with a substantial amount of mRNA for HA-Filamin A and GFP. In contrast, HA-Filamin A protein was effectively accumulated in the presence of calpeptin, an inhibitor of the Ca^{2+}-dependent protease calpain (Fig. 4). These results suggested that degradation of Filamin A was activated by calpain or an equivalent calpeptin-sensitive protease(s) in the presence of FILIPs. We next examined whether the degradation of Filamin A could be affected by its other binding partner F-actin, and HA-tagged Filamin A lacking the actin-binding domain (HA-ΔABD-Filamin A; the actin-binding domain of Filamin A corresponds to N-terminal 274 residues) was used instead of HA-Filamin A. Then, we found that the relative amount of HA-ΔABD-Filamin A to GFP was not reduced in the presence of L-FILIP compared with that in the absence of L-FILIP (Fig. 4; Nagano *et al.*, 2002), suggesting that L-FILIP preferentially induces degradation of F-actin-associated Filamin A. Because Filamin A exerts its functions in F-actin dynamics (Stossel *et al.*, 2001), this seems to be a reasonable feature as a Filamin A-regulating protein.

Filamin A-interacting proteins 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 results in human cortical malformation in which affected post-mitotic cells remain in the ventricular zone (Eksioglu *et al.*, 1996; Fox *et al.*, 1998), it is likely that FILIPs play a pivotal role in the control of cell migration in the developing neocortex. To examine the role of FILIPs on neuronal migration *in vivo*, we introduced FILIP cDNAs into the E18 rat neocortex by electroporation, so that a fraction of the 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 labeled in the ventricular zone migrated out towards the pial surface (Fig. 5A,B). These migrating cells were spindle-shaped with leading and trailing processes oriented radially and identified as neurons (Hatanaka & Murakami, 2002). In contrast, cells expressing S-FILIP–GFP or L-FILIP–GFP were quite different in shape and motility from those labeled with GFP only, being round, devoid of long and radially oriented processes and hardly migrating (Fig. 5A,B). These 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 (Y. Hatanaka, unpubl. obs., 2001).

Degradation of Filamin A in the ventricular zone

Filamin A-interacting proteins, especially L-FILIP, were expressed dominantly during the late embryonic period when corticogenesis is in progress (Fig. 6A). Although predominant expression of Filamin A protein in the migrating and post-migratory neurons in the intermediate zone and cortical plate of human embryonic brain has been demonstrated (Fox *et al.*, 1998), our *in situ* hybridization histochemical study showed robust expression of *Filamin A* mRNA in the ventricular zone, as well as in the

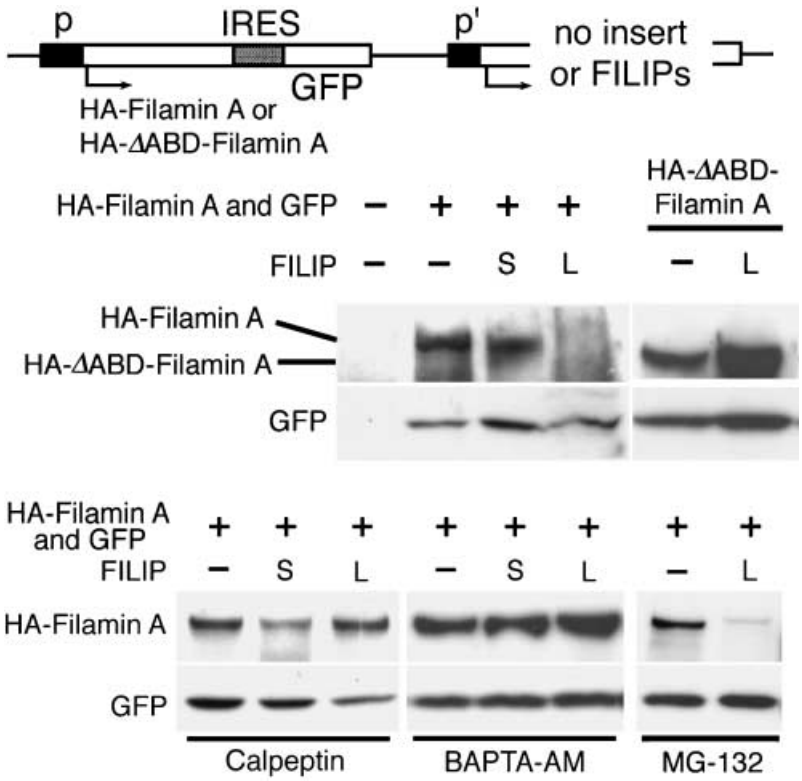

Figure 4. Filamin A-interacting proteins (FILIPs) induce degradation of Filamin A. Recombinant Filamin A and FILIPs were coexpressed in the same COS-7 cell. The upper drawing shows the structure of the vector used. An internal ribosome entry site (*IRES*) sequence was inserted between the *HA-tagged Filamin A* (*HA-Filamin A*) and *green fluorescent protein* (*GFP*) cDNAs. These cDNAs were driven by a cytomegalovirus promoter (p), whereas *FILIPs* were expressed under the elongation factor (EF)-1α promoter (p′). The 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 in the presence of either short-form (S-) FILIP (S) or long-form (L-) FILIP (L) could be estimated based on the expression of GFP by immunoblotting using anti-HA and anti-GFP antibodies, respectively. Relative amounts of HA-Filamin A, but not those of HA-Filamin A lacking the actin-binding domain (ΔABD-Filamin A), expressed at approximately 48 h after transfection were decreased in the presence of FILIPs, especially L-FILIP (middle panels). However, these effects were diminished in the presence of either calpeptin (50 μmol/L), a calpain inhibitor, or BAPTA-AM (20 μmol/L), an intracellular Ca^{2+}-chelating reagent, suggesting that FILIPs induced degradation of Filamin A (lower panels). The proteasome inhibitor MG-132 (10 μmol/L) had no obvious effect. (Modified from Nagano *et al.*, 2002.)

cortical plate (Fig. 6C; Nagano *et al.*, 2002). Because *FILIPs* mRNA was expressed in the ventricular zone, it is likely that FILIPs interact with Filamin A and induce its degradation there. Indeed, our immunoprecipitation experiment revealed that endogenous FILIP (mainly L-FILIP) interacted with endogenous Filamin A in the E12 rat cortex (Fig. 6B) and a large number of cells in the ventricular zone of E16 rat exhibited less immunoreactivity for Filamin than cells in the intermediate zone and cortical plate (Fig. 6D; Nagano *et al.*, 2002). These results support the notion that FILIP induces degradation of Filamin A in the ventricular zone. Based on the data presented so far, we concluded that the novel molecule FILIP exerts its effects through a Filamin A–F-actin axis to control the start of neocortical cell migration from the ventricular zone (Nagano *et al.*, 2002).

ΔABD-Filamin A inhibits radial migration through the subventricular and intermediate zones to the cortical plate in the developing mouse cerebral cortex *in vivo*

We next investigated the significance of Filamin A during radial migration *in vivo* (Nagano *et al.*, 2004). We used *in utero* electroporation-mediated gene transfer for mice (Inoue & Krumlauf, 2001; Saito & Nakatsuji, 2001; Tabata & Nakajima, 2001), which allows the delivery of plasmids into ventricular zone cells and post-mitotic migrating neurons of embryonic neocortices *in utero*. Because cortices subjected to a sham operation continued to develop normally, this method enabled us to evaluate the net effect of the injected plasmid as much as possible. Using this method and a Filamin A mutant without the actin-binding domain (ΔABD-Filamin A) that suppresses Filamin A activity (Kainulainen *et al.*, 2002), we introduced an enhanced GFP (EGFP; control) or EGFP-tagged ΔABD-Filamin A (EGFP-ΔABD-Filamin A) expression plasmid into E15 mouse dorsolateral neocortices *in vivo* and labeled cells were observed at E17 (day 2 post-transfection) or E19 (day 4 post-transfection). On day 2, EGFP- or EGFP-ΔABD-Filamin A-labeled cells were mostly found in the ventricular zone and SVZ/IZ. We could not detect any inhibitory effect of the mutant Filamin A against the early phase of radial migration up to the SVZ/IZ, because similar percentages of EGFP-ΔABD-Filamin A- and EGFP-labeled cells were present outside the ventricular zone (see Nagano *et al.*, 2004). In contrast, these labeled cells showed distinct distribution patterns on day 4 (Fig. 7A,B). Although a substantial number of EGFP-labeled cells was found in the cortical plate, and many seemed to have completed radial migration

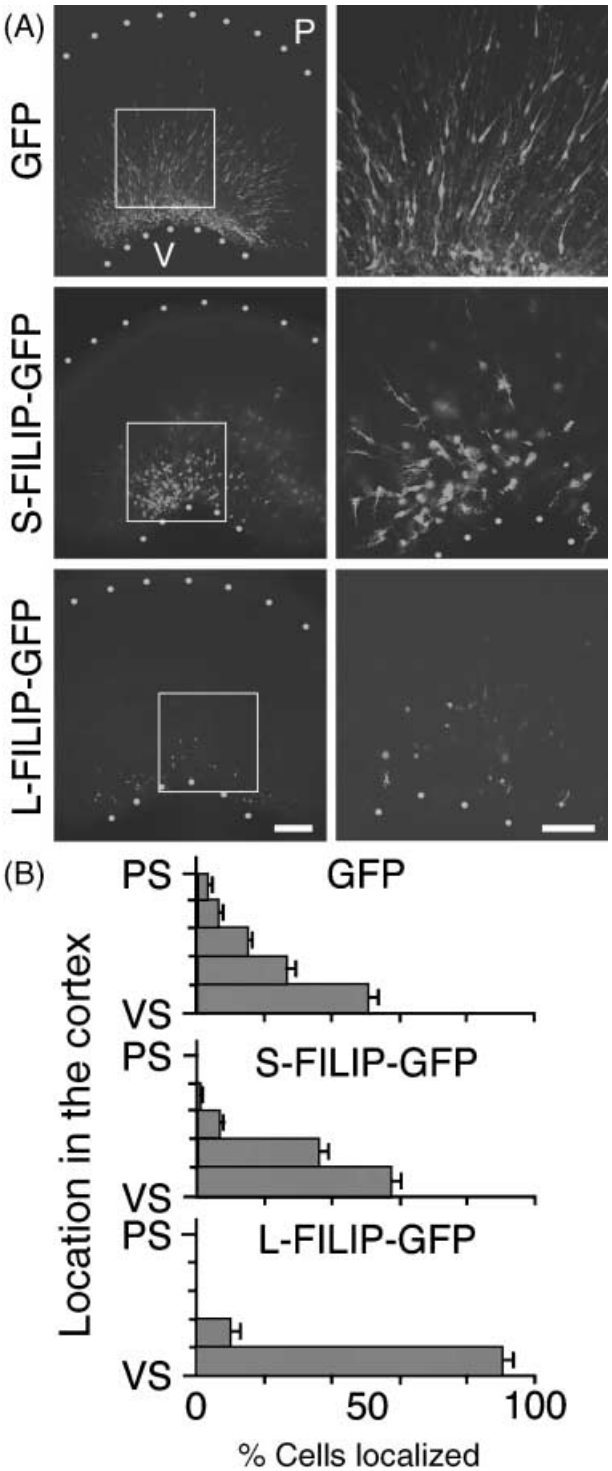

Figure 5. Filamin A-interacting proteins (FILIPs) suppress radial cell migration in neocortical explants. (A) The cDNAs of green fluorescent protein (GFP; upper panels), short-form (S-) FILIP-GFP (middle panels) or long-form (L-) FILIP-GFP (lower panels) were introduced electroporatically into the ventricular zone of embryonic day 18 rat cortex. Dissected cortices were maintained as explants for 4 days. Radially migrating post-mitotic neurons were labeled with GFP, whereas most cells expressing S- or L-FILIP-GFP were round and remained close to the ventricular zone. Right column shows higher-magnification views of each square in the left column. Grey dots indicate the edges of the slices. P, pial surface; V, lateral ventricle. Bars, 200 μm (left column); 100 μm (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-labeled 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). Data are the mean ± SEM. (Modified from Nagano *et al.*, 2002.)

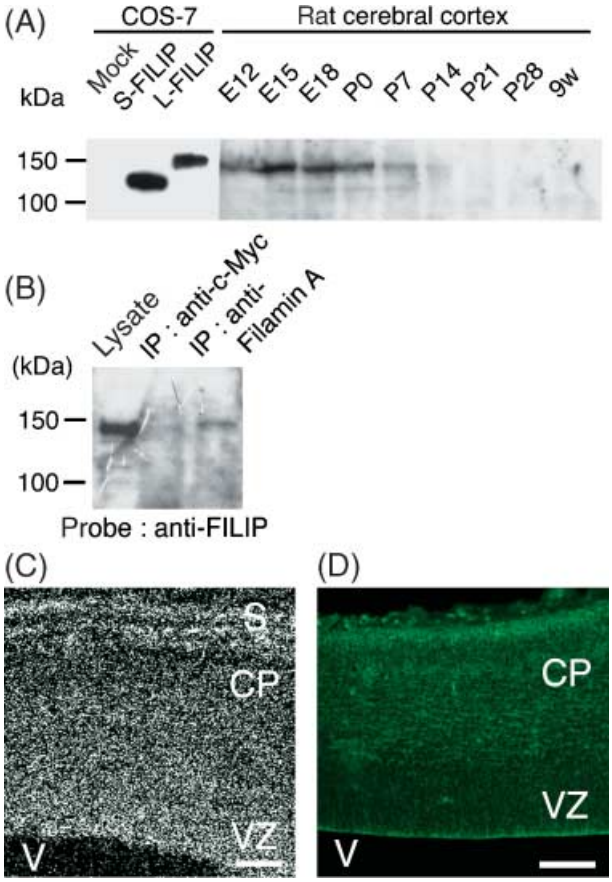

Figure 6. Long-form Filamin A-interacting protein (L-FILIP) dominantly interacts with Filamin A in the developing neocortex. (A) Ontogenetic expression profiles of L-FILIP and short-form (S-) FILIP in the developing rat neocortex were analyzed by immunoblotting. Long-form FILIP was more dominant in amount than S-FILIP. Anti-FILIP antibody recognized both S- and L-FILIP expressed in COS-7 cells with high specificity. (B) Long-form FILIP was found in the lysate of embryonic dat (E) 12 rat neocortices, whereas S-FILIP was hardly detected (left lane). Long-form FILIP was coimmunoprecipitated with anti-Filamin A antibody from the same lysate (right lane), whereas anti-c-Myc antibody did not give any positive signal (middle lane; control). (C) *In situ* hybridization histochemistry using E16 rat neocortex showed that expression of the *Filamin A* gene was observed throughout the developing cortex, with strong expression in the ventricular zone (VZ). CP, cortical plate; S, skull; V, lateral ventricle. Bar, 100 μm. (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)). V, lateral ventricle. Bar, 100 μm. (Modified from Nagano *et al.*, 2002.)

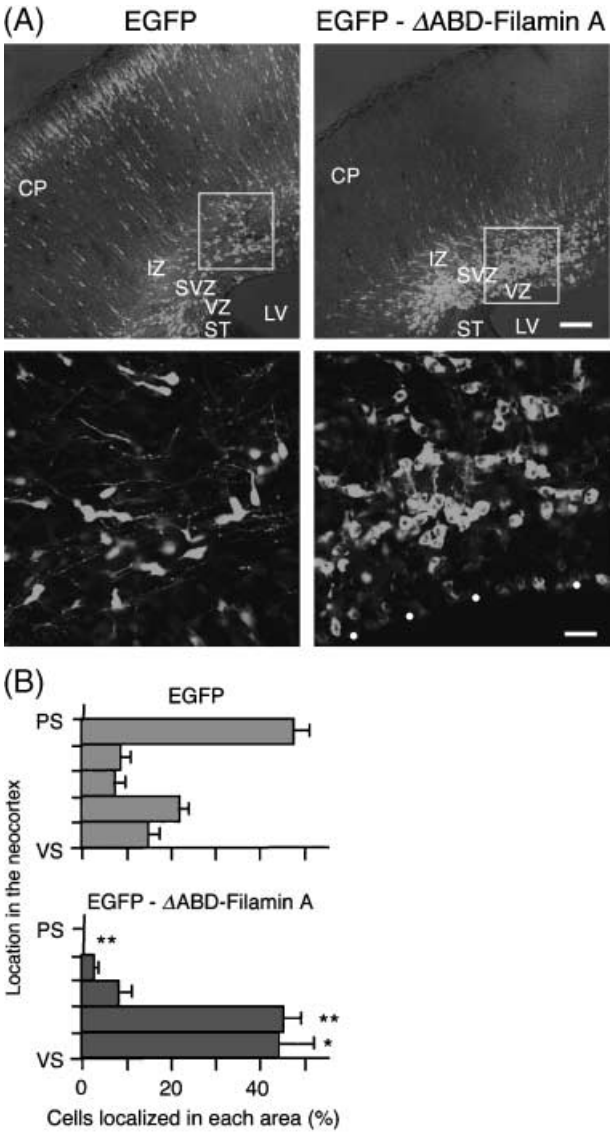

Figure 7. Filamin A is required for radial migration through the subventricular and intermediate zones *in vivo*. (A) Enhanced green fluorescent protein (EGFP) or EGFP-ΔABD-Filamin A was expressed in mouse dorsolateral neocortices *in vivo* using electroporation-mediated gene transfer on embryonic day (E) 15. Labeled cells were observed on E19 (day 4). The EGFP- and EGFP-ΔABD-Filamin A-labeled cells (left and right columns, respectively) exhibited different distributions and appearances. Differential interference contrast images are merged for the upper panels. The lower panels show higher-magnification views of the areas indicated by the squares in the upper panels. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; LV, lateral ventricle; ST, striatum. Bars, 100 μm (upper panels); 25 μm (lower panels). (B) Quantitative analyses of cell migration in (A). The neocortex was subdivided evenly into five areas from the ventricular surface (VS) to the pial surface (PS). The percentages of labeled cells (cell bodies) in each area were calculated for three independent experiments for each group. Data are the mean ± SEM. Asterisks indicate that these percentages are significantly different from those of the same areas in EGFP-transfected cortices (*$P < 0.05$, **$P < 0.01$, *t*-test). (Modified with permission of the Society for Neuroscience from Nagano *et al*., 2004.)

based on their location, most of the EGFP-ΔABD-Filamin A-labeled cells were still in the SVZ/IZ. These results suggest that Filamin A is required for radial migration through the SVZ/IZ (or into the cortical plate) *in vivo*.

ΔABD-Filamin A causes aberrant regulation of cell polarity in the subventricular and intermediate zones

In the SVZ/IZ of the developing neocortex, many neurons exhibit multipolar migration, during which they do not have any fixed polarity as they do during locomotion or somal translocation in the cortical plate (Tabata & Nakajima, 2003). The neurons move irregularly and slowly, with their multiple neurites extending and retracting dynamically. Therefore, the finding that mutant Filamin A prevented neuronal migration through the SVZ/IZ prompted us to focus on the neurites of cells in the SVZ/IZ that expressed the mutant.

We observed the morphology of the EGFP- or EGFP-ΔABD-Filamin A-expressing cells in the SVZ/IZ on days 2 and 4 after electroporation *in utero* at E15. On day 2, both the EGFP- and EGFP-ΔABD-Filamin A-labeled cells showed multipolar morphology and their cell bodies and processes tended to be oriented tangentially (see Nagano *et al*., 2004), as described previously (Tabata & Nakajima, 2003). On day 4, the EGFP-labeled cells found in the SVZ/IZ showed a similar morphology to that observed on day 2 (Fig. 7A). In contrast, the EGFP signals from the EGFP-tagged mutant Filamin A-expressing cells on day 4 generally indicated a round shape and a lack of neurites (Fig. 7A), suggesting that these cells had lost the neurites they had possessed 2 days before.

The next question was whether the mutant Filamin A affected neurite dynamics in the SVZ/IZ cells before the neurites were lost and how this effect related to cellular motility. To address these issues, we performed time-lapse observations of cells expressing EGFP-tagged mutant Filamin A plus EGFP or EGFP only in the dorsolateral neocortex 2 days after *in utero* electroporation on E15. In the control experiment, during which only EGFP was expressed, many SVZ/IZ cells showed dynamic extension and retraction of multiple neurites in various directions while migrating slowly and irregularly in a radial direction (Fig. 8A; see movie in Nagano *et al*., 2004; http://www.jneurosci.org/content/vol24/issue43/images/data/9648/DC1/Supplemental_movie_S1.mov), as reported previously (Tabata & Nakajima, 2003). In addition, they sometimes took on an almost bipolar shape and temporarily moved faster (Fig. 8A; see movie in Nagano *et al*., 2004; http://www.jneurosci.org/content/vol24/issue43/images/data/9648/DC1/Supplemental_movie_S1.mov). The SVZ/IZ cells containing the mutant Filamin A also actively extended and

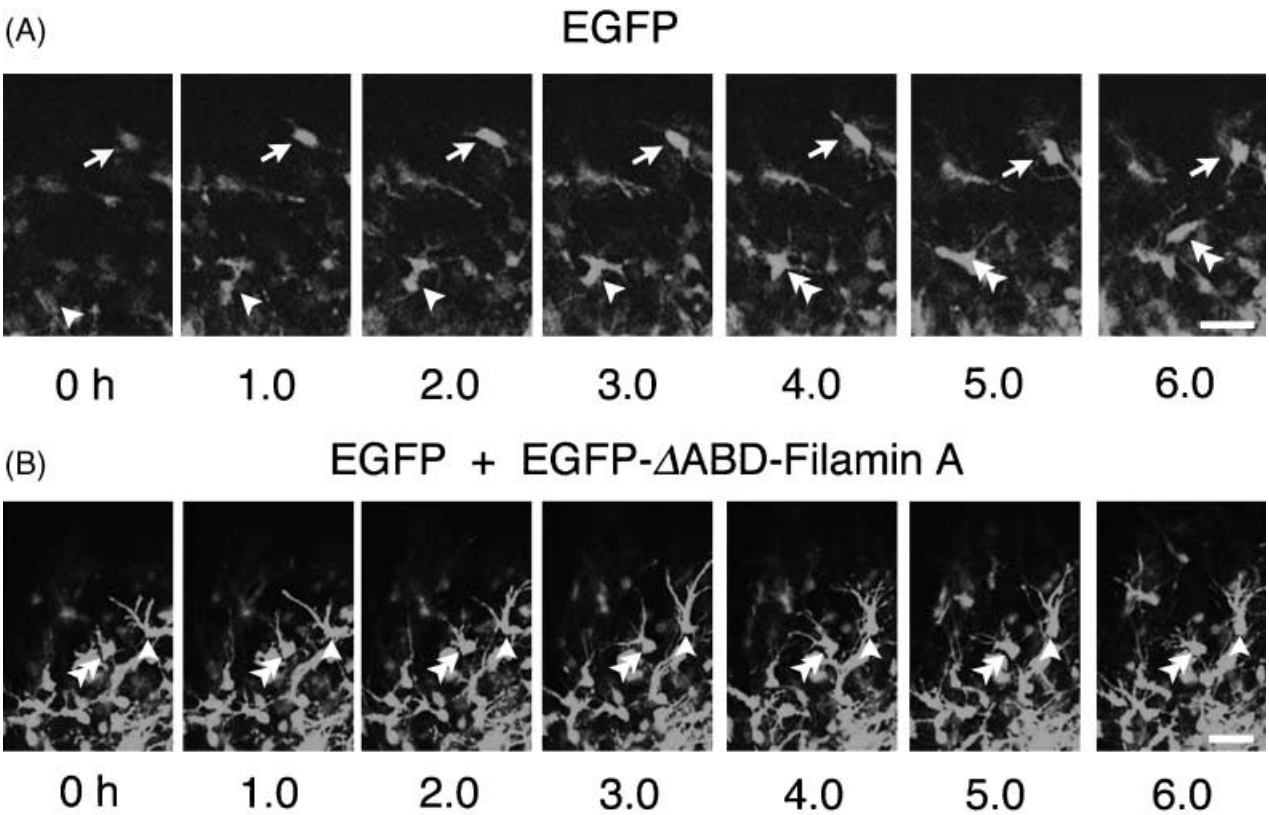

Figure 8. Antimigratory effect of ΔABD-Filamin A on cells in the intermediate zone is due to aberrant regulation of cell polarity. (A) Enhanced green fluorescent protein (EGFP) or (B) EGFP-ΔABD-Filamin A plus EGFP was expressed in mouse dorsolateral neocortices *in vivo* using electroporation-mediated gene transfer on embryonic day (E) 15. Labeled cells were analyzed in living slices on E17 (day 2). (A) The EGFP-expressing cells in the intermediate zone exhibited typical multipolar migration, during which they advanced slowly and irregularly while dynamically extending and retracting multiple processes (arrow and arrowhead). They sometimes took on an almost bipolar shape and temporarily moved faster (double arrowheads; the same cell as indicated by the single arrowhead at 0–3.0 h). (B) The ΔABD-Filamin A-expressing cells also actively and repeatedly extended and retracted multiple neurites. However, the neurites tended to show more complicated branching and the cells hardly exhibited a bipolar shape or intermittent rapid motion (single and double arrowheads). The pial surface is to the top in each panel. Bars, 25 μm. (Modified with permission of the Society for Neuroscience from Nagano *et al.*, 2004.)

retracted multiple neurites. However, the neurites tended to show more complicated branching and the cells hardly ever exhibited a bipolar shape or intermittent rapid motion (Fig. 8B; see movie in Nagano *et al.*, 2004; http://www.jneurosci.org/content/vol24/issue43/images/data/9648/DC1/Supplemental_movie_S2.mov). The observation of the EGFP-ΔABD-Filamin A-labeled cells also showed active dynamics of apparently short neurites. Therefore, the mutant Filamin A seemed to primarily affect cell polarity rather than disrupt neurite dynamics during the cell rounding process in the SVZ/IZ.

Filamin A overexpression caused by FILIP dysfunction also affects cell polarity in the subventricular and intermediate zones

Our *in vivo* results show that dysfunction of Filamin A affects the regulation of cell polarity, eventually making cells in the SVZ/IZ round (Figs 7A,8B). These observations led us to hypothesize that the amount of Filamin A present during the early phases of radial migration (i.e. up to the IZ) was responsible for cell shape during multipolar migration through the SVZ/IZ. To test this, we attempted to increase the Filamin A content of radially migrating cells *in vivo* by coexpressing FILIP siRNA-3 with EGFP through electroporation *in utero* on E15. We had confirmed that *FILIP* siRNA-3 was effective to knock-down recombinant FILIP expression in COS-7 cells, whereas *FILIP* siRNA-2 was not. Therefore, *FILIP* siRNA-2 served as a control in the experiments described below (see Nagano *et al.*, 2004). First, we examined the amount of Filamin A protein in neocortices expressing the siRNA on day 2 after electroporation and confirmed that greater accumulation of Filamin A occurred in FILIP siRNA-3-expressing neocortices than in FILIP siRNA-2 (control siRNA)-expressing neocortices

(Fig. 9A). We next compared the morphologies of the IZ cells expressing the siRNA on day 2. The results showed that FILIP siRNA-3 caused some of the cells to take on a radial orientation and bipolar spindle shape, whereas multipolar cells typical of those normally seen in the IZ were found predominantly in neocortices expressing FILIP siRNA-2 (control siRNA; Fig. 9B,C). These results suggest that appropriate expression of Filamin A by cells undergoing radial migration through the SVZ/IZ, which is at least partially regulated by FILIP, is responsible for controlling their polarity to characterize multipolar migration.

Because the radially oriented spindle-shaped cells induced in the IZ by FILIP siRNA-3 showed a similar morphology to those undergoing locomotion or somal translocation in the cortical plate, we next investigated whether their behavior resembled typical migration in the cortical plate. We performed time-lapse observations of IZ cells expressing FILIP siRNA-2 or -3 together with EGFP in the dorsolateral cortex 2 days after *in utero* electroporation on E15. The behavior of control cells (those expressing FILIP siRNA-2) was essentially the same as that observed in the neocortices expressing EGFP only. In contrast, the IZ cells containing FILIP siRNA-3, which exhibited

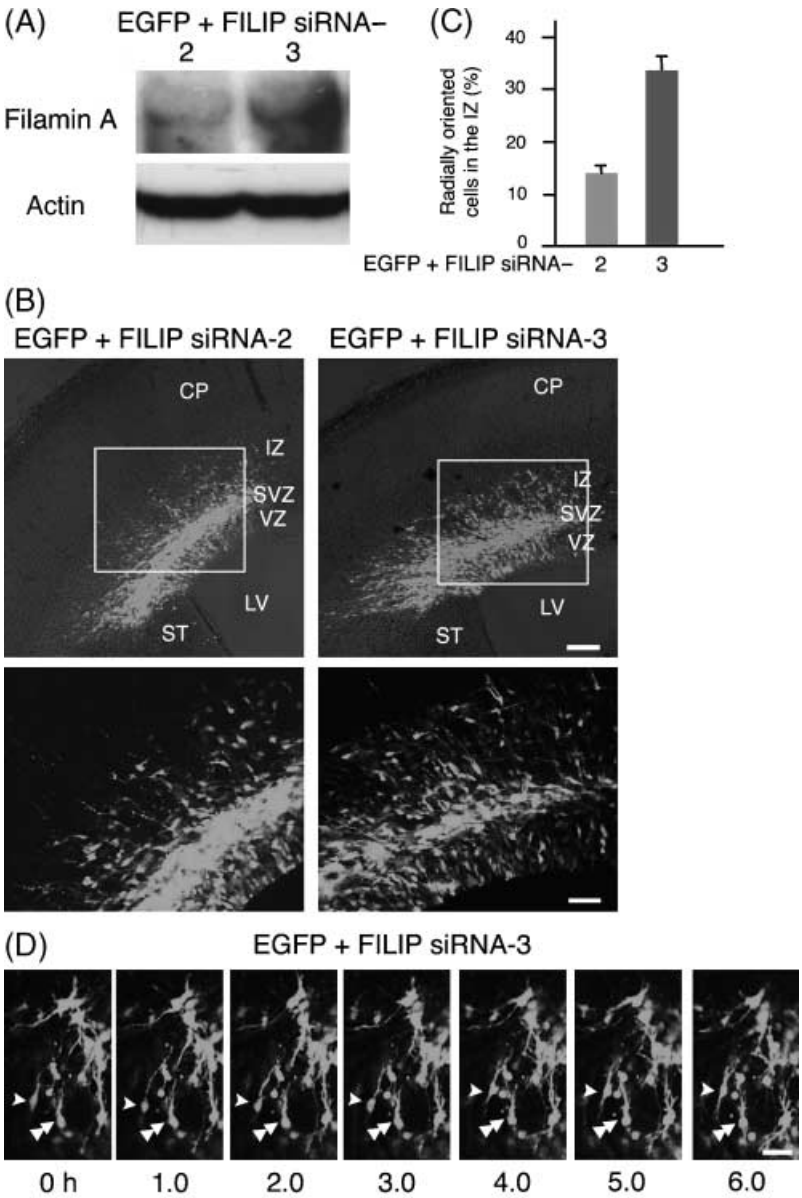

Figure 9. Overexpression of Filamin A in cells of the intermediate zone restricts cell polarity to the radial direction, rather than modifies neurite dynamics. Filamin A-interacting protein (FILIP) short interfering RNA (siRNA)-2 or -3 was expressed together with enhanced green fluorescent protein (EGFP) in mouse dorsolateral neocortices *in vivo* using electroporation-mediated gene transfer on embryonic day (E) 15 and was analyzed on E17 (day 2). (A) Immunoblot analysis confirmed that FILIP siRNA-3 increased Filamin A expression (upper panel). Filamin A-interacting protein siRNA-2, which had little effect, served as a negative control, whereas expression of actin served as loading controls (lower panel). (B) Intermediate zone cells containing FILIP siRNA-3 (right column) tended to show a radially oriented spindle shape. Those containing FILIP siRNA-2 (left column) served as controls. Differential interference contrast images are merged for the upper panels. The lower panels show higher-magnification views of the areas indicated by the squares in the upper panels. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; LV, lateral ventricle; ST, striatum. Bars, 100 μm (upper panels); 25 μm (lower panels). (C) Quantitative analyses of cell shape in the intermediate zone. Radially oriented spindle-shaped cells (defined as those with a long axis/short axis ratio of > 2.5) were counted and their percentage relative to the total number of labeled cells in that region was calculated for three independent experiments in each group. Data are the mean ± SEM. There was a significant difference between the groups ($P < 0.01$; *t*-test). (D) Electroporated brain tissues were cut on E17 and labeled cells were analyzed in living slices (time-lapse observation). Many FILIP siRNA-3-expressing cells in the intermediate zone maintained their polarity during the observation period, unlike control cells that exhibited multipolar migration. However, they repeatedly extended and retracted their neurites in consistent radial directions (arrowhead and double arrowheads). The pial surface is to the top in each panel. Bar, 25 μm. (Modified with permission of the Society for Neuroscience from Nagano *et al.*, 2004.)

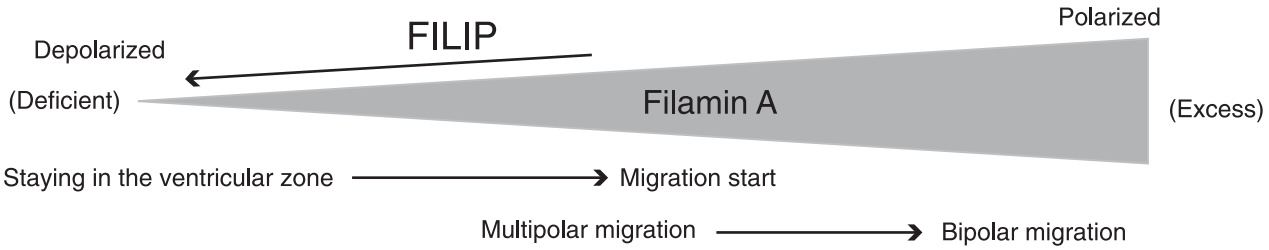

Figure 10. The amount of Filamin A is critical for radial migration. Filamin A plays an important role for the start of migration and migratory cell shape in an amount-dependent manner. Filamin A-interacting protein (FILIP) reduces the amount of Filamin A by enhancing its degradation.

a radially oriented spindle shape, behaved in a distinct manner (Fig. 9D; see movie in Nagano *et al*., 2004; http://www.jneurosci.org/content/vol24/issue43/images/data/9648/DC1/Supplemental_movie_S3.mov). Most maintained their radial polarity during the observation period; however, their two major neurites (leading and trailing processes) did not remain still, but were repeatedly extended and retracted in consistent radial directions. These results suggest that hyperactivity of Filamin A in the SVZ/IZ cells restricts the cell polarity to radial direction rather than modifying neurite dynamics. Therefore, FILIP seems to prevent SVZ/IZ cells from acquiring the fixed polarity to enable multipolar migration.

Concluding remarks

We identified a novel Filamin-interacting protein, FILIP, through our search for genes in the ventricular zone expressed more abundantly when most postmitotic neurons were just about to migrate than when many neurons had already left (Nagano *et al*., 2002). FILIP negatively controlled the function of Filamin A and *FILIP* mRNA was expressed in ventricular zone cells, but not in migrating or post-migratory cells. These findings describe a novel molecular basis of FILIP and Filamin A to regulate the start of neocortical cell migration from the ventricular zone (Schuldt, 2002). Furthermore, we obtained evidence indicating that Filamin A is involved in regulating cell polarity and motility in the SVZ/IZ during radial migration (Nagano *et al*., 2004). We investigated this issue using a mutant Filamin A molecule to inhibit Filamin A function and by knocking-down *FILIP* mRNA to enhance the activity of Filamin A. Our results show that cells with suppressed Filamin A function have difficulty in acquiring consistent polarity and establishing a bipolar form in the SVZ/IZ, whereas Filamin A overexpression increases the number of bipolar cells. These results suggest that Filamin A is essential for regulating the polarity of neocortical neurons during radial migration and that the actin cytoskeleton (on which Filamin A resides) is important for radial migration (Fig. 10). Because Filamin A is known to interact with various proteins, including cell surface receptors and signal transduction molecules (Stossel *et al*., 2001), we hope that further studies, especially on the subcellular localization of Filamin A, help to reveal the signaling cascades of polarity control of radially migrating cells, disclose the underlying molecular events of cell behavior control and unveil the mystery of corticogenesis in near future.

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