 Human Molecular Genetics, 2002, Vol. 11, No. 23 2845–2854

Filamin A and Filamin B are co-expressed within neurons during periods of neuronal migration and can physically interact

Volney L. Sheen[1,†], Yuanyi Feng[1,†], Donna Graham[1], Toshiro Takafuta[2], Sandor S. Shapiro[2] and Christopher A. Walsh[1,3,*]

[1]Division of Neurogenetics, Department of Neurology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115, USA, [2]Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania 19107, USA and [3]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02115, USA

Received June 21, 2002; Revised August 17, 2002; Accepted August 26, 2002

Mutations in the X-linked gene Filamin A (*FLNA*) lead to the human neurological disorder, periventricular heterotopia (PH). Although PH is characterized by a failure in neuronal migration into the cerebral cortex with consequent formation of nodules in the ventricular and subventricular zones, many neurons appear to migrate normally, even in males, suggesting compensatory mechanisms. Here we characterize expression patterns for FlnA and a highly homologous protein Filamin B (FlnB) within the nervous system, in order to better understand their potential roles in cortical development. *FlnA* mRNA was widely expressed in all cortical layers while *FlnB* mRNA was most highly expressed in the ventricular and subventricular zones during development. In adulthood, widespread but reduced expression of *FlnA* and *FlnB* persisted throughout the cerebral cortex. FlnA and FlnB proteins were highly expressed in both the leading processes and somata of migratory neurons during corticogenesis. Postnatally, FlnA immunoreactivity was largely localized to the cell body with FlnB in the soma and neuropil during neuronal differentiation. In adulthood, diminished expression of both proteins localized to the cell soma and nucleus. Moreover, the putative FLNB homodimerization domain strongly interacted with itself or the corresponding homologous region of FLNA by yeast two-hybrid interaction, the two proteins co-localized within neuronal precursors by immunocytochemistry and the existence of FLNA–FLNB heterodimers could be detected by co-immunoprecipitation. These results suggest that FLNA and FLNB may form both homodimers and heterodimers and that their interaction could potentially compensate for the loss of FLNA function during cortical development within PH individuals.

INTRODUCTION

Periventricular heterotopia (PH) is a neurological disorder characterized by the failure of a subset of neurons to migrate into the cerebral cortex during development (1–3). This migrational arrest results in the formation of nodules, composed of neurons, which line the ventricular and subventricular zones. The heterotopia lie beneath a normal appearing cerebral cortex that seems to function quite well. Recent studies have demonstrated that PH can result from mutations in the X-linked gene, Filamin A (*FLNA*) (4–7). *FLNA* encodes a large (280 KD) actin-binding protein with additional domains that interact with multiple cellular proteins, membrane receptors, thereby providing potentially crucial links from receptor signal transduction to the actin cytoskeleton (8,9). Typically, patients with PH are heterozygotes for *FLNA* mutations, since male embryos with *FLNA* mutations seem to be miscarried before birth. X-inactivation of either the normal or mutated X-chromosome is assumed to account for the divergent behavior of normally migrated and heterotopic neurons, although this hypothesis has not been tested experimentally.

Recent genetic mutational analysis, however, has demonstrated that occasional males with PH can also present with *FLNA* mutations, suggesting that random X-inactivation alone cannot account for the differential behavior of normal and heterotopic neurons (6,7). The males with PH appear to have partial loss of

*To whom correspondence should be addressed at: Room 816, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA, USA. Tel: +1 6176670813; Fax: +1 6176670815; Email: cwalsh@caregroup.harvard.edu
†The authors wish it to be known, that in their opinion, the first two authors should be regarded as joint First Authors.

function *FLNA* mutations (e.g., Leu→Phe at amino acid 656 and Tyr→stop at amino acid 2305) and thus, they may possess a partially functional FLNA protein which might permit normal fetal development. On the other hand, males with *FLNA* mutations show neurons with either complete arrest of migration or normal migration. Furthermore, no evidence of mosaicism was detected for these mutations, implying that normally and abnormally located neurons express the same allele (6). These data suggest that some other compensatory mechanism that does not rely on X-inactivation probably exists and allows some neurons expressing the mutant FLNA protein to migrate.

FLNA belongs to a family of actin-binding proteins, of which only one other member, Filamin B (FLNB), is expressed within the central nervous system and shares approximately 70% amino acid sequence homology with FLNA (10). Given the high degree of similarity between these two proteins, we sought to characterize the temporal and spatial patterns of FLNA and FLNB expression, thereby providing additional insight into their potential roles in neuronal migration and differentiation. Because of the extensive temporal and spatial overlap seen in this expression data and the known ability of FLNA to form homodimers, we also tested whether FLNA and FLNB might heterodimerize. Such interactions would offer another potential mechanism by which neurons expressing the aberrant FLNA protein could interact with a functional FLNB protein and complete a normal migratory process.

We found that both actin-binding proteins are expressed in the cell body and leading process of migratory neurons during periods of cortical development. Protein expression is greatest within neuronal precursors and is downregulated with neuronal differentiation. The spatial distribution also changes at the onset of differentiation with greater localization of FlnA and FlnB to the soma and the soma with neuropil, respectively. Nuclear staining of both filamin proteins was apparent in terminally differentiated neurons. At the mRNA level, FlnA appears to be widely distributed during development, including the vasculature, but has the greatest level of overlapping expression with FlnB within the ventricular and subventricular zones. Furthermore, the carboxy terminus of FLNA, which normally supports FLNA homodimerization, can bind with FLNB in a yeast two-hybrid assay. Co-localization of the two proteins was appreciated on immunocytochemistry. Co-immunoprecipitation of FLNB by FLNA also confirmed this interaction. These results raise the possibility that heterodimerization of FLNA to FLNB may play a role in neuronal migration.

RESULTS

Filamin A and Filamin B expressions are downregulated following neuronal differentiation *in vitro*

We examined the temporal levels of expression of the filamin actin-binding proteins to assess at which stage they were more likely to influence cortical development. Western blots taken from whole mouse brain extracts suggest a subtle decrease in FlnA and FlnB expression in adulthood as compared to tissue obtained from embryonic day 16 (E16) to postnatal day 15 (P15) mouse brain (time points representative of periods of ongoing neuronal proliferation and differentiation) (Fig. 1A and B).

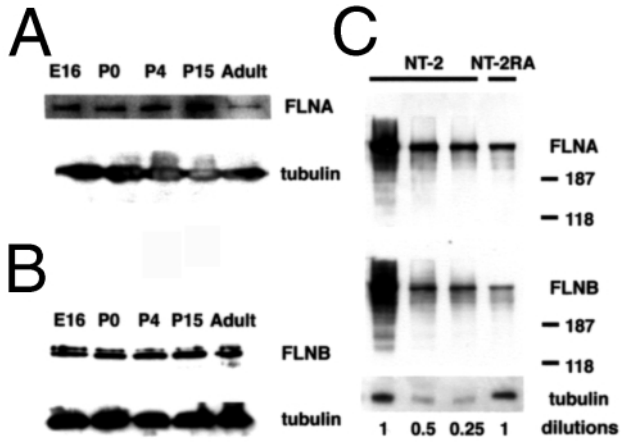

Figure 1. Filamin A and Filamin B are developmentally regulated during corticogenesis. (**A** and **B**) Western blot analysis of whole brain mouse extracts taken at various stages of development suggests a down regulation in FlnA and FlnB protein levels during adulthood, respectively. (**C**) This trend is more apparent within pure NT-2 EC neuronal and neuronal precursor populations. Higher levels of FLNA and FLNB expression are found in the precursor population (NT-2 EC) as compared to the differentiated neuronal population (NT-2RA). Tubulin loading controls and serial dilutions (1, 0.5, 0.25 for NT-2 EC and 1 for NT-2RA) demonstrate equal loading in the first and fourth lanes.

To examine a purified neural population, protein levels were examined in the human NT-2 EC cell line, prior to and after differentiation with retinoic acid. Previous studies have shown the cell line to be representative of a restricted neural precursor population with the capacity to undergo terminal neuronal differentiation (11–13). Western blot analysis from these samples showed a dramatic down-regulation of both FLNA and FLNB within differentiated neurons as compared to the precursor population (Fig. 1C). The overall level of FLNA expression appeared greater than that observed for FLNB.

Expression of Filamin A and Filamin B messenger RNA throughout developing cerebral cortex

Given that both filamin proteins are highly expressed within neuronal precursors during periods of ongoing cortical development, we then examined whether the spatial patterns of expression were consistent with a role in affecting neuronal migration and proliferation. Messenger RNA levels were evaluated in embryonic, early postnatal, and adult mouse cortices.

Throughout the developing embryo (E16), FlnA and FlnB have significant but distinct expression patterns as seen by *in situ* hybridization (Fig. 2). FlnA showed highest levels of detectable signal within blood vessels, renal cortices, respiratory and alimentary tracts, olfactory epithelium and presumed isles of hematopoesis within the liver. Within the cortex, RNA message was seen more clearly in the ventricular zone and cortical plate, as compared to the intermediate zone, although this observation could partially reflect the cell densities in the respective layers. A lower but widespread level of message was seen in the nervous system, including the embryonic choroid plexus. It is unclear whether this signal localizes to the choroid plexus epithelium or intervening vasculature. FlnB mRNA expression was most apparent in the periventricular region of

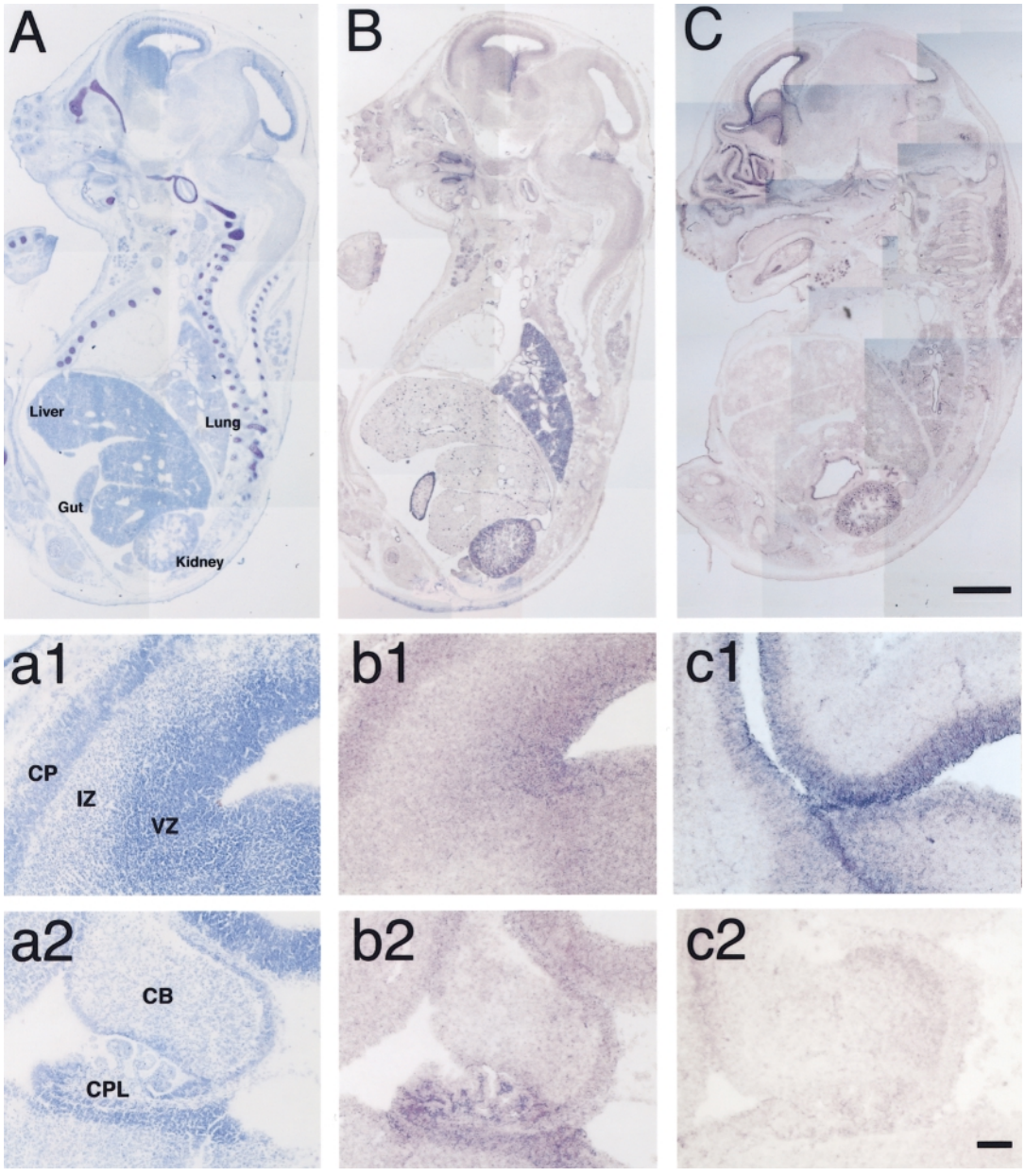

Figure 2. FlnA and FlnB are expressed in various mouse organ systems during development. Sagittal brightfield photomicrographs of an embryonic day 16 (E16) mouse following (**A**) Cresyl violet staining, (**B**) FlnA and (**C**) FlnB *in situ* hybridization reveal significant expression of both filamin mRNA throughout the embryo. FlnA shows highest levels of signal detected in the vasculature, the alveoli of the lungs, renal cortex, alimentary tract, olfactory epithelium and presumed isles of hematopoesis in the liver. Widespread but lesser levels of expression are seen in the cerebral cortex and choroid plexus. FlnB mRNA can be found in the periventricular region of the brain and the olfactory epithelium and to a lesser degree, in the airways of the lung, renal cortices and alimentary tract. (**a1** and **a2**) Higher magnifications of the insets in (A) represent the developing forebrain and cerebellum, respectively. The corresponding adjacent sections (**b1** and **b2**) and (**c1** and **c2**) demonstrate the mRNA expression for FlnA and FlnB. Within the central nervous system, FlnA and FlnB appear most abundant in the developing forebrain. FlnB mRNA localizes further and preferentially to the embryonic subventricular and ventricular zones (VZ) and to a lesser degree, to the embryonic cortical plate (CP) and intermediate zone (IZ). An elevated level of FlnA mRNA is also detected within embryonic choroid plexus (CPL), adjacent to the cerebellar anlage (CB) (Scale bars in A,B,C = 1 mm; a–c = 100 μm).

the brain and olfactory epithelium with less intense labeling in the kidneys, lung, and alimentary tract.

Filamin mRNA expression within the central nervous system complemented the pattern seen with western blot analysis (Fig. 3). At E12.5, both FlnA and FlnB mRNA were seen in the developing ventricular and subventricular zones. At E14.5 and E16.5, expression of FlnA was widely distributed across the width of cerebral cortex whereas FlnB showed predominant staining of the periventricular region, with greater expression in the forebrain as compared to more caudal structures. A similar pattern was present but diminished in early postnatal mice. Finally, increased FlnA and FlnB labeling was seen in the

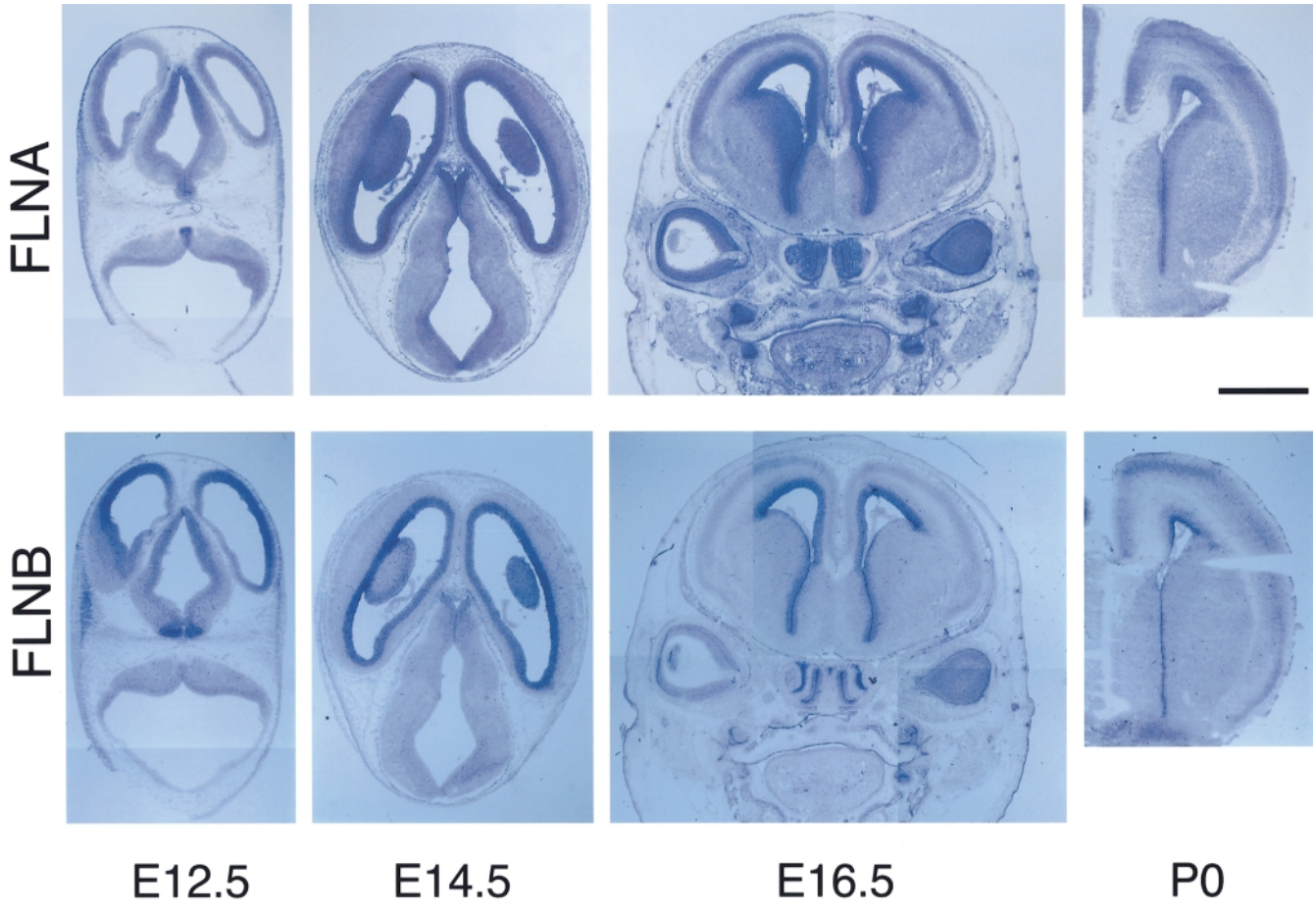

Figure 3. Expression of FlnA and FlnB messenger RNA in mouse cerebral cortex at various ages of development. Coronal brightfield photomicrographs following *in situ* hybridization with FlnA or FlnB messages show labeling within the ventricular zone at E12.5, with increasing expression at E14.5 and E16.5 and somewhat diminished expression at P0 (coronal section through one hemisphere). FlnA mRNA appears widely distributed across the cortical mantle, whereas FlnB mRNA localizes predominantly to the ventricular and subventricular zones during development. FlnA and FlnB levels are also more robust in the forebrain as compared to the hindbrain (Scale bar = 1 mm).

Purkinje cells of the cerebellum, although lower levels of both filamin mRNA generally persisted throughout the adult CNS (Fig. 4). The widespread expression of FlnA as compared to the more restricted expression of FlnB suggested that FlnA may be more involved in fundamental and numerous processes of cortical development, with FlnB playing a more selective role during the period of neurogenesis.

Expression of Filamin A and Filamin B protein throughout the developing cerebral cortex

In order to examine the distribution of FlnA and FlnB in the central nervous system in greater detail, we performed immunohistochemical localization studies.

On embryonic day 16 (E16), immunohistochemistry for both FlnA and FlnB showed robust staining within the entire cell soma and leading processes of migratory neurons (Fig. 5A, E16). FlnA immunoreactivity was relatively uniform between the cortical plate (CP) and ventricular zone (VZ). FlnB demonstrated a similar pattern, although there appeared to be greater staining within processes, including radial glial fibres (Fig. 5B, E16, arrow). There was also increased FlnB protein staining along the wall of the lateral ventricles (VZ). Within early postnatal mice (P0, P7), the cellular localization shifted during ongoing neuronal differentiation, with FlnB staining most apparent in the apical dendrites and cell soma, while FLNA localized primarily to the cell soma (Fig. 5, P7, arrows). Finally, both FlnA and FlnB displayed predominantly somatic and apparent nuclear staining within adult cerebral cortex (Fig. 5, Adult, arrowheads).

To confirm the presence of both Filamin A and Filamin B within neurons, immunocytochemical staining for both the filamin proteins and neuronal markers was performed *in vitro* (Fig. 6). Neurons staining for either TUJ1 or MAP2 (data not shown) clearly showed immunoreactivity using antisera specific for Filamin A or Filamin B in culture. Furthermore, in early postnatal cultured pyramidal neurons, Filamin B immunoreactivity localized more extensively to cell soma and neuronal processes, whereas Filamin A immunoreactivity had greater expression in the cell soma alone. Both filamin proteins also showed some nuclear staining. This same complementary pattern of staining for the filamin proteins was seen *in vivo* for postnatal mice during the period of ongoing neuronal differentiation.

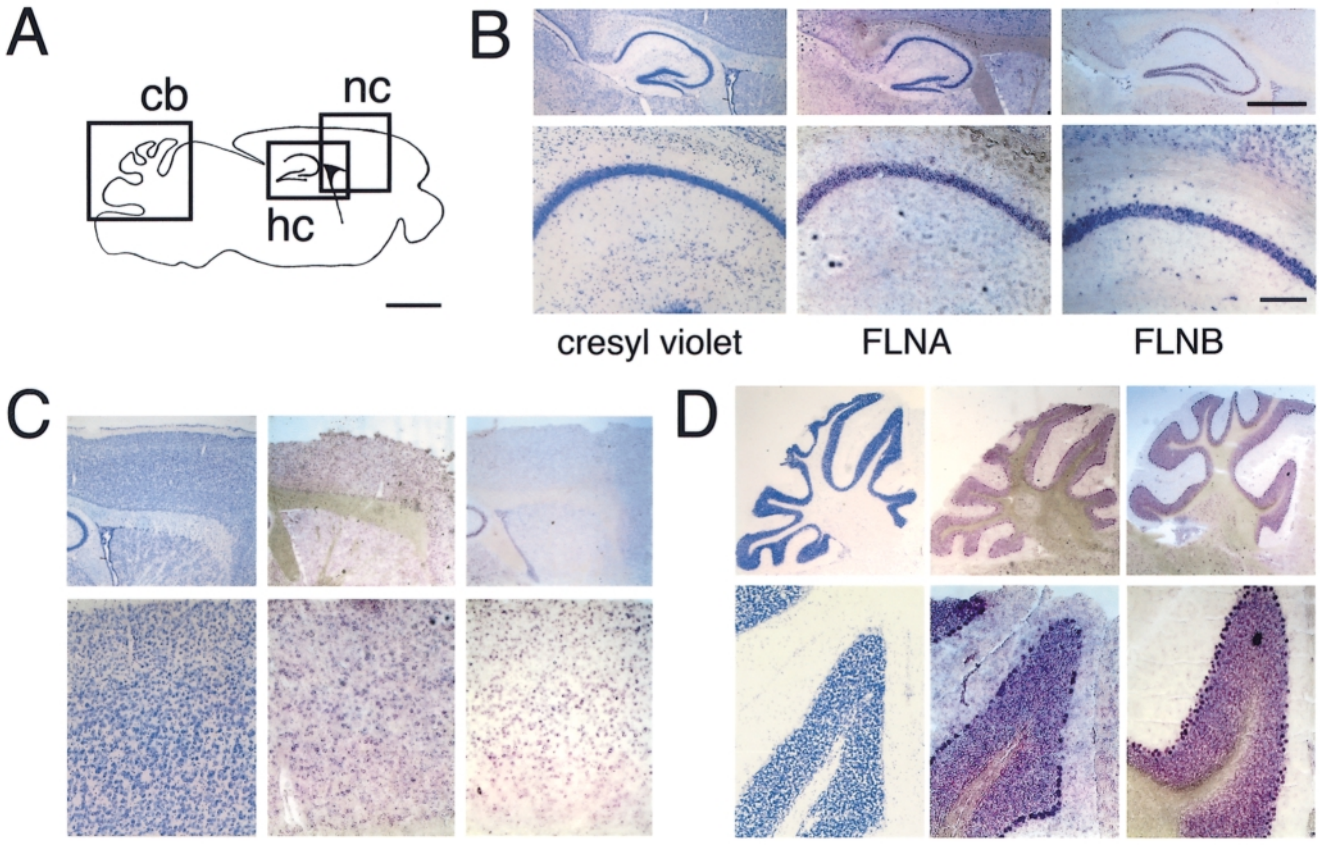

Figure 4. Low level expression of FlnA and FlnB mRNA within adult CNS. (**A**) Camera lucida drawing of sagittal adult mouse brain with corresponding photomicrographs in (**B**–**D**), representing the neocortex (nc), hippocampus (hc) and cerebellum (cb). (B,C) Nissl stains, FlnA and FlnB *in situ* hybridizations reveal low level filamin expression within neurons in the adult hippocampus and cerebral cortex, respectively. (D) Both FlnA and FlnB localize to the cerebellar purkinje cells, but diminished and ubiquitous expression of mRNA also exists throughout the granule cell layer (Scale bars in A = 1 mm; B–D = 200 μm).

FLNA and FLNB can interact to form heterodimers

Given the extensive temporal and spatial overlap in expression between the two filamin proteins, we sought to evaluate the potential binding interaction of FLNB to FLNA. The yeast two-hybrid assay was employed using FLNA and FLNB LexA fusion constructs, consisting of the C-termini amino acids 2167–2647 and 2122–2602, respectively (Fig. 7A). Introduction of the VP-16 FLNB clones (aa 2507–2602 and aa 2300–2602) into the L40 yeast cells, containing either the LexA FLNA or LexA FLNB constructs, led to activation of the LacZ reporter. The VP-16 FLNB fragments include the putative binding domain, repeat 24, for the filamin proteins. Increased β-galactosidase activity was also appreciated with the FLNA–FLNB interactions as compared with FLNB–FLNB binding (Fig. 7B).

Additionally, increased β-galactosidase activity was seen with the FLNB VP-16B construct compared to the FLNB VP-16A construct in binding to the FLNB LexA bait. This increased affinity suggested that repeats 16–23 between hinge 1 and hinge 2 could also participate in filamin heterodimerization. To further address this observation, VP-16 FLNB fragments (aa 2071–2225 and aa 2060–2212) corresponding to these regions were designed and tested for interaction with LexA FLNA or LexA FLNB in a yeast two-hybrid assay. Activation of the LacZ reporter in these constructs suggests another binding domain distinct from repeat 24 (Fig. 7B).

Although the binding of FLNB repeat 24 to itself appears strong in the two-hybrid assay, the interaction between FLNB to FLNA showed equal or greater strength, suggesting the potential for heterodimerization. Both FLNA and FLNB also co-localized to the cell soma within NT-2 EC cells, which represent a neuronal precursor-like cell population (Fig. 7C). This overlapping spatial expression pattern might allow for potential binding interactions and the formation of heterodimers. Finally, to look into this possibility, immunoprecipitation was performed using a monoclonal antibody that is specific for FLNA. FLNB was present in the FLNA immunocomplex from the NT-2 EC cell extracts, demonstrating that the two proteins can indeed form heterodimers in cultured cells, and thus could potentially interact *in vivo* as well (Fig. 7D).

DISCUSSION

The human neurological disorder PH can be caused by mutations in the *FLNA* gene. Within the cerebral cortex of such affected individuals, however, a significant number of neurons assume appropriate laminar positions, suggesting that

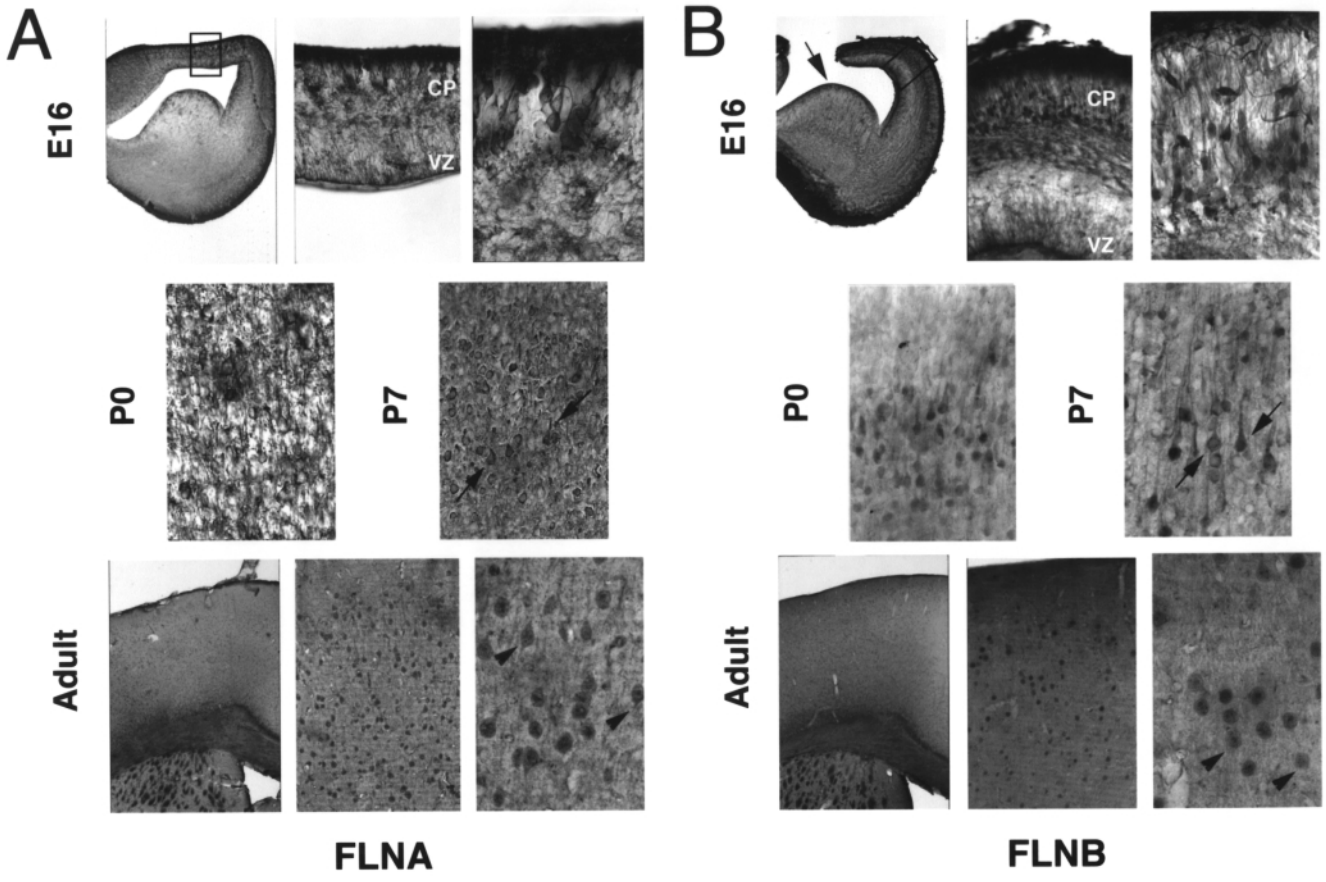

Figure 5. Protein expression of FlnA and FlnB in both embryonic, early post-natal and adult cerebral cortex suggests shared and distinct functional roles across various stages of development. (**A**) Photomicrographs of FlnA immunostaining at embryonic days 16 (E16), post-natal day 0, 7 (P0, P7) and adulthood within murine cerebral cortex shows initial filamin expression within the cell bodies and leading processes of migratory neuroblasts. FlnA expression diminishes post-natally with staining most apparent in the cell soma. Subsequently within adult mice, FlnA localizes to the cell soma and nucleus of cortical neurons (arrowheads). (**B**) Photomicrographs of FlnB immunostaining at various developmental stages within murine cerebral cortex largely demonstrates a similar pattern seen with FlnA. However, FlnB protein appears more abundant along the surface of the lateral ventricle in the embryonic cerebral cortex, as well as cell processes including radial glia. FlnB expression diminishes post-natally, but can be appreciated within the dendrites of cortical pyramidal neurons (P0 and P7, arrows). Within adult mice, FlnB labeling is found in the cell soma and nucleus of cortical neurons (arrowheads). Thus, FlnA and FlnB do have subtle differences in patterns of expression, suggesting different developmental roles (CP = cortical plate, VZ = ventricular zone).

additional compensatory processes can direct the ultimate spatial fate of a given neuroblast. Prior studies have suggested that factors including X-inactivation within heterozygous females and the severity of the *FLNA* mutation likely influence this developmental sequence. The present studies extend these findings by suggesting a potential physical interaction between the FLNA and FLNB proteins in neuronal proliferation and migration. Expression of both filamin mRNAs is highest during periods of ongoing neurogenesis and neuronal migration in the mouse (E14–E16). FlnA mRNA is widely distributed across the cortical mantle, but also overlaps with the more restricted expression of FlnB mRNA within the ventricular and subventricular zones, the regions of periventricular heterotopia formation. Although clearly showing differences in distribution patterns, both filamin proteins are found abundantly within neuronal precursors and within migratory neurons. Lastly, FLNA and FLNB have the capacity to undergo heterodimerization, as evidenced by both the yeast two-hybrid interactions and co-immunoprecipitation. They also co-localize on immunocytochemical staining. Given the temporal and spatial overlap, this interaction raises the possibility that FLNA–FLNB heterodimers may compensate for dysfunctional FLNA homodimers, thereby permitting neuroblast migration from the ventricular zone into the cortical plate.

The differential localization of FlnA and FlnB expression suggests that they have both shared and distinct roles in the central nervous system during development and adulthood. Appropriate for their presumed role in migration, both filamin proteins are abundantly expressed within the cell soma and leading process of migratory neurons during cortical development. FlnB mRNA and protein, however, appear more highly localized to the periventricular region of the forebrain, perhaps implicating a greater role during neuronal proliferation, while FlnA has a widespread pattern of expression, consistent with roles across multiple stages of development. Subsequently, in early postnatal mice, FlnA resides predominantly within the cell soma whereas FlnB localizes more to the soma and neuropil. This observation raises the possibility that FlnB may

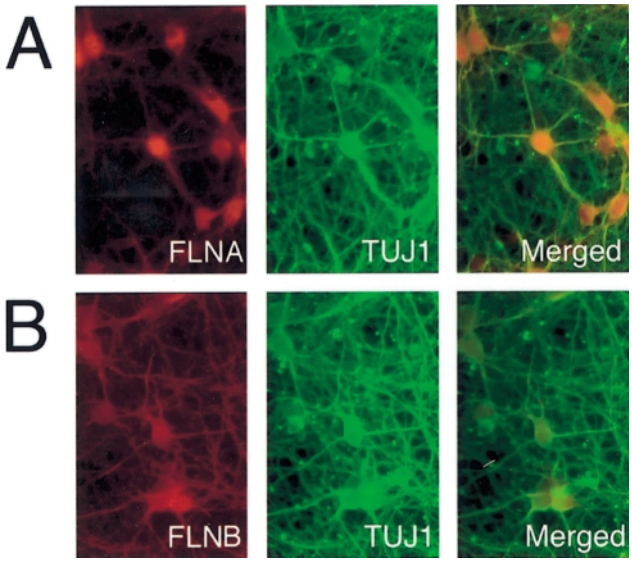

Figure 6. Filamin A and Filamin B are found within neurons. (**A**) Photomicrographs of double labeling for FlnA and the neuronal specific marker TUJ1, respectively, confirm expression of the protein in primary rat neurons. Superimposition of the images demonstrates staining within the same cells (far right). (**B**) Photomicrographs of double labeling for FlnB and the neuronal specific marker TUJ1, respectively, confirm expression of the protein in primary rat neurons. Superimposition of the images demonstrates expression within the same cells (far right). Additionally, FlnB localizes more extensively to the neuronal processes as compared to FlnA, suggesting a greater role in neurite support or extension.

also be more involved in process outgrowth, consistent with prior observations that FLNB binds to presenilin within neuropil threads and dystrophic neurites (14). Finally, the somatic and nuclear localization of both filamin proteins in adulthood may indicate a function in cellular maintenance and regulation of nuclear transcription factors (15) (unpublished observations, Feng, Sheen and Walsh). Although the filamin proteins are present from the early stages of cortical development into adulthood, the varying patterns of expression argue for both shared and distinct contributions to neuronal migration, differentiation and maintenance.

While the mechanism by which *FLNA* mutations result in PH remains speculative, prior reports suggest that filamin is involved in cellular motility. The FLNA protein directly interacts with the actin cytoskeleton and is known to regulate cellular stability, protrusion and motility across various biological systems (8) (16–18). From the current studies, both FlnA and FlnB expression appear more pronounced in the developing brain with slightly lower levels detected in adulthood. A much greater difference is observed in a purified neural precursor population as compared to terminally differentiated neurons within the NT-2 EC cell line. The developmental regulation of filamin would be consistent with a role for the actin-binding proteins in the proliferation and/or migration of neuronal precursors. Thus, mutations in FlnA could actually disrupt the intrinsic machinery necessary for neurons to migrate, leading to heterotopia along the ventricles (19). Alternatively, some studies have suggested an extrinsic cause of neuronal heterotopia through disruption of radial glial fibers (20) or local vascular changes. Filamin A clearly is expressed in the blood vessels and hematopoetic cells, raising the possibility of local disruption of blood flow along the ventricles and subsequent formation of PH. Whether PH arises from a primary disruption of intrinsic neuronal migration or secondary vascular insufficiency remains to be seen.

The capacity to form FLNA–FLNB heterodimers is not surprising given the high degree of homology between the two proteins. Excluding the two hinge regions, FLNA and FLNB share 70% sequence homology, although they are entirely dissimilar in the 1st hinge and only 44% identical in the 2nd hinge (10). Prior studies have also predicted that the likely FLNA dimerization domain lies within repeat 24 on the basis of hydrophobicity and charge and have gone further to show that recombinant protein corresponding to this region can homodimerize (8,9,21). The current studies show that the two proteins can interact in this same C-terminal region containing repeat 24 by the yeast two-hybrid screen. The co-immunoprecipitation confirms this interaction within a neuronal precursor cell line. Furthermore, the two-hybrid studies suggest that repeats between the two hinge regions may also serve as binding domains, providing additional points of contact between the two filamin proteins. This observation would be consistent with prior studies, indicating that FLNA heterodimers may exist in two distinct conformational states. The two proteins may bind at the terminal end of the receptor-binding region (repeat 24 alone) thereby forming a 'V' conformation with mobility around hinge 2. Alternatively, they may form a 'Y' conformation with heterodimerization involving repeats 16–24 and mobility around hinge 1 (8,9,21). Finally, the intensity of β-galactosidase activity suggests that the FLNA–FLNB interaction may actually be stronger than that observed between FLNB and FLNB. Taken in the context of recent findings showing that different splice variants of FLNA and FLNB provide differential regulation of myogenesis (22), these observations raise the likelihood that different combinations of these actin-binding proteins will modulate the actin cytoskeleton and presumably, neuronal migration, either directly or indirectly.

FLNA and FLNB interactions provide another potential alternative mechanism giving rise to a normal appearing cerebral cortex within individuals with PH. Mutations in the *FLNA* gene presumably disrupt signal transduction from the filamin receptor-binding region down to the actin-binding domains, which modulate the cytoskeleton. Analysis of the human X-linked *FLNA* mutations further indicates that truncation of the C-terminus (the last 342 amino acids) or single point mutations (L656F) are sufficient to cause PH in males (6). Even in such male patients with a single, apparently mutated allele of the *FLNA* gene, however, the cerebral cortex appears relatively intact, implying that many neurons are able to migrate normally. Since the filamin proteins are structurally similar, can interact, and co-localize, *FLNB* may provide an alternate receptor-binding domain absent in the dysfunctional *FLNA* protein and thereby permit appropriate signal transduction to the actin cytoskeleton. Such compensation could explain the phenotype in the male individual with the single point mutation (L656F). Although the second male mutation (Tyr to Stop at aa 205) lacks the putative binding domain in repeat 24, the potential binding domains between the two hinge

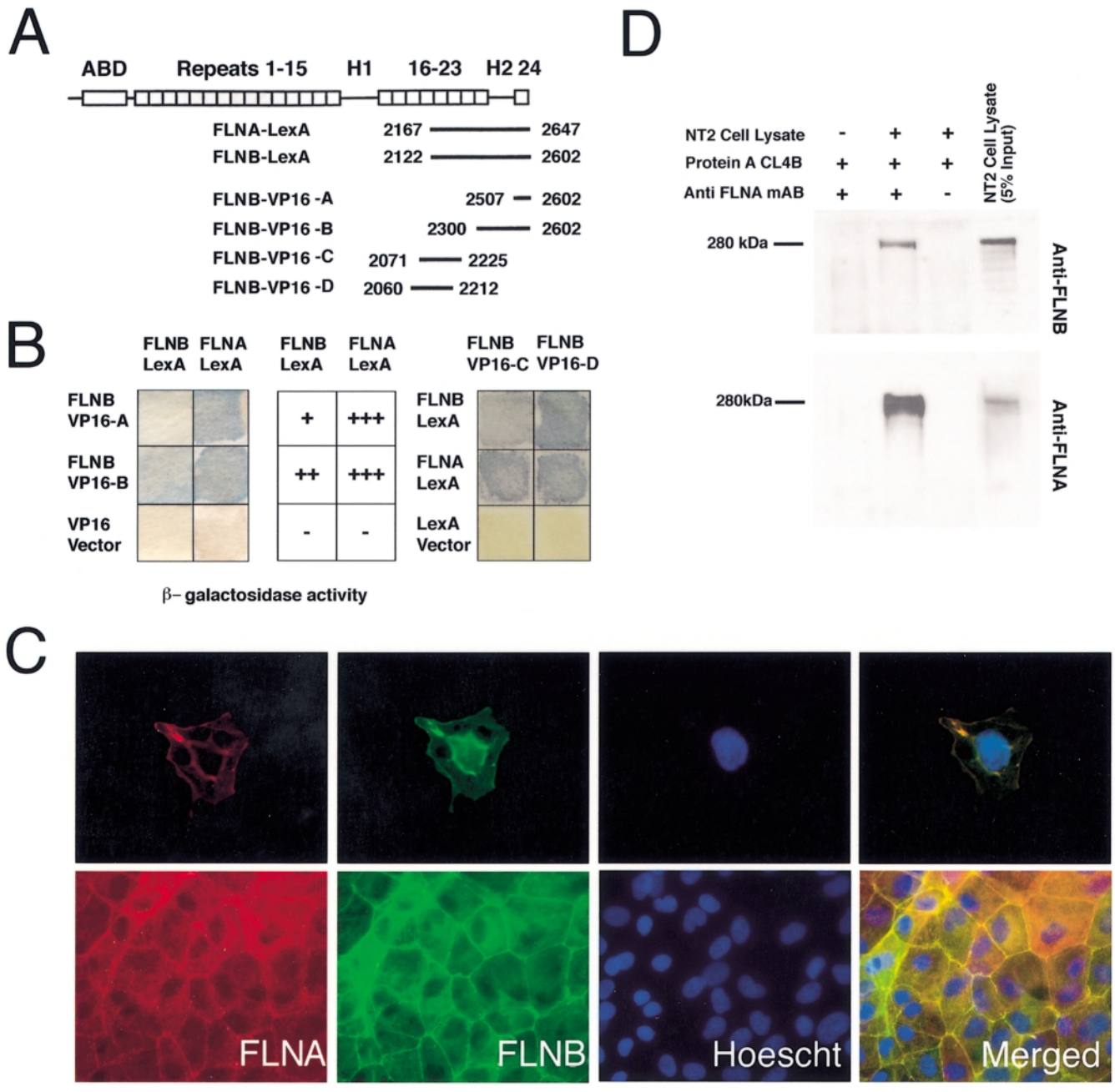

Figure 7. Interaction of Filamin A and Filamin B. (**A**) Yeast two-hybrid LexA constructs for FLNA and FLNB utilize the last 480 amino acids of the receptor binding region, including repeat 24 and the 2nd hinge region (aa 2167–2647 and aa 2122–2602, respectively). Four FLNB VP16 clones (aa 2507–2602, aa 2300–2602, aa 2071–2225 and aa 2060–2212) were transformed into L40 yeast cells containing the LexA FLNA or FLNB construct (H1 = hinge 1, H2 = hinge 2). (**B**) Filter assay demonstrates activation of LacZ reporter gene following binding of VP16-FLNB-A/B, containing repeat 24, to the LexA FLNA/FLNB. The corresponding extent of β-galactosidase activity is summarized (+++ > ++ > + > −). Filter assay also demonstrates activation of LacZ reporter gene following binding of VP16-FLNB-C/D, corresponding to repeats between the two hinge regions. (**C**) Photomicrograph of a single human NT-2 EC neuronal precursor cell is stained for FLNA, FLNB, and nuclear Hoescht, respectively (left to right). The composite, merged images reveal significant overlap of FLNA and FLNB primarily within the cell cytoplasm. Photomicrographs of a confluent NT-2 EC culture stained for FLNA, FLNB, and nuclear Hoescht (left to right). The merged fluorescent images (far right) again show significant overlap of the filamin proteins within the cell cytoplasm, with most pronounced staining at the cell junctions and periphery in these neural precursors. (**D**) Co-immunoprecipitation demonstrating pull down of FLNB by Anti-FLNA antibody within the human NT-2 EC cell lysate.

regions could permit functional interactions. Alternatively, heterodimerization of *FLNB* with a dysfunctional *FLNA* may be sufficient to permit signaling of other filamin-binding regulatory molecules including SAP kinase, Trio and the Rho and Ras GTPases and thereby influence cell migration (23–25). Finally, *FLNB* homodimerization alone may provide some degree of compensation. This possibility is consistent with the observation that *FLNA* null melanoma cells can recover partial

mobility with overexpression of *FLNB* (26). Thus, FLNA homodimers, FLNB homodimers and FLNA–FLNB heterodimers may all play some role in effecting neuronal heterotopia formation.

In summary, the overlapping distribution and expression of FLNA and FLNB suggests functional redundancy or complementation of the two genes and proteins. The potential heterodimerization of the two filamin isoforms further supports a potential functional overlap of FLNA and FLNB. Taken in the context of the human neurologic disorder PH, the interaction of FLNA and FLNB may provide another mechanism to allow for proper neuronal migration.

METHODS AND MATERIALS

Animals

The study includes Swiss-Webster (Taconic) and C57bl/6J mice (Jackson Labs) and Charles River Sprague Dawley rats from our institutional colony. They were housed and treated in accordance with protocols approved by the IACUC of Harvard Medical School.

Tissue culture

Primary rat cultures, or human cell lines (NT-2 EC cell line, Stratagene, La Jolla, CA, USA) were cultured by previously described methods (27). Briefly, cells were grown in DMEM or DMEM/F12 (Gibco, Carlsbad, CA, USA), 10–15% v/v fetal calf serum and 1% penicillin-streptomycin depending on the cell line. Primary rat cells were maintained on tissue cultures dishes (Corning, Acton, MA, USA) pretreated with poly-1-lysine in a 37°C/5% CO_2 incubator. The NT-2 EC cells were maintained and passaged per the NT-2 EC culture protocol (Stratagene). Neuronal differentiation of the human neuronal restricted precursor cell line was performed by supplementation of the media with retinoic acid (10 μM) and subsequent mitotic inhibitors, as directed (11–13).

Western blot analysis

Protein was extracted from both mice brains and the human NT-2 EC cell lines by previously described methods (28). Briefly, tissue was solubilized in lysis buffer, separated on a 7.5% SDS-PAGE gel and transferred onto PVDF membrane. Truncated Filamin A protein served as control for the Filamin B antibodies. Membranes were probed with anti-FLNA and anti-FLNB antibodies and detected by enhanced chemiluminescence. Antibodies against Filamin A were obtained commercially Chemicon, Temecula, CA, USA (mouse and human), and Novacastra, Newcastle Upon Tyne, UK (human). A rabbit polyclonal antibody to Filamin B was prepared as previously described (10). A7 and M2 lines (courtesy Dr T. Stossel), melanoma cells with and without Filamin A, respectively, served as controls (data not shown). Antibodies to Filamin A and Filamin B did not cross-react.

Immunohistochemistry

The appropriately aged mice were perfused with saline and fixative (2% paraformaldehyde in phosphate buffered saline (PBS)). Coronal sections (50 μm) were cut on a vibratome, permeabilized with Tween 20 and washed. Cell cultures were washed and fixed with methanol (−80°C). Samples were placed in blocking solution with PBS containing 10% fetal calf serum, 5% horse serum and 5% goat serum, incubated overnight in the appropriate antibody (TUJ1, GFAP, MAP2: Sigma, St. Louis, MO, USA; FLNA, FLNB), and processed through standard avidin/biotin amplification (Vectastain, Burlingame, CA, USA) or fluorescent secondaries (CY3, Jackson Immunoresearch Laboratories, Westgrove, PA, USA, and FITC, Sigma). Antibodies directed against FLNA were used to evaluate expression in human NT-2 EC cell cultures (FLNA antibodies obtained from Novacastra), rat and mice neuronal cultures, and mice brain (FLNA antibodies from Chemicon or reagents courtesy of Dr Stossel). A rabbit polyclonal antibody to Filamin B was prepared as previously described (10).

In situ hybridization

In situ hybridization was performed according to previously described methods (29). The probes were obtained from linearized FlnA and FlnB cDNA templates. The two FLNA oligoprimers contained nucleotides 5′-CAGACCTTAGC-CTACTCACAGCC-3′ and 5′-ACTGATCTTCACAGTGAAT-GGGC-3′. The two FLNB oligoprimers contained nucleotides 5′-GATGATAACAGACGCTGCTCCC-3′ and 5′-GCCTGCA-TCTCTTGTGT-3′. Appropriate controls using the sense strands were performed.

Yeast two-hybrid screen

The carboxyl terminals of the human FLNA and FLNB proteins (amino acids 2167–2647 and 2122–2602, respectively) were fused with the LexA vector and screened against FLNB fragments, which had been inserted into a pVP16 transactivation domain (30). The FLNB fragments (amino acids 2122–2602 and 2300–2602) contain repeat 24 of the filamin protein, which is thought to represent the putative homodimerization domain. The FLNB fragments (amino acids 2071–2225 and amino acids 2060–2212) contain repeats 19–21 between the two hinge regions. The LexA FLNA and FLNB 'baits' were obtained by PCR amplification of the corresponding cDNA. Given the significant size of the genes, the LexA FLNA and FLNB 'baits' chosen correspond to the receptor-binding regions of filamin, where interactions are most likely to occur. The resulting PCR products were cloned into the pcrTOPOII plasmid (Invitrogen), sequenced and then subcloned into PMBT116 to form an in-frame fusion of LexA. The VP-16 fusion plasmid was modified from previously described methods (30). All LexA and VP16 fusion constructs were transformed into the yeast L40 strain via standard techniques using PEG and lithium acetate. The transformants were grown under proper selection and hybrid interaction was determined by a filter-based β-galactosidase assay (31). The respective filamin LexA and VP-16 constructs showed no auto-activation (data not shown).

Co-immunoprecipitation

NT2 cells were grown to 95% confluence and lysed in 100 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, 20 mM NaF, 1 mM

DTT and 0.07% Triton × 100 with 20 µg/ml leupeptin, 20 µg/ml pepstatin A, 10 mM bezamidin and 1 mM PMSF.

To perform immunoprecipitation, appoximately 3 µg of NCL-FIL monoclonal anti-FLNA antibody (Novocastra) was added to 2 mg cell lysate and incubated at 4°C overnight with 15 µl of Protein A conjugated sepharose (Phamarcia). The immunocomplexes bound to Protein A sepharose were washed 6 times in the lysis buffer, then eluted with SDS sample buffer (30 mM Tris pH 6.8, 0.1% SDS, 350 mM beta mecaptoethonal and 10% glycerol) and analyzed on 7.5% SDS-PAGE followed by immunoblotting with antibodies indicated.

ACKNOWLEDGEMENTS

The authors would like to thank Drs T. Stossel and D. Kwiatkowski for their generosity in supplying the Filamin A antibodies. We additionally thank Dr U. Berger for his assistance and expertise in performing the *in situ* hybridizations. This work was supported by grants to C.A.W. from the NINDS (5RO1 NS35129 and 1PO1NS40043) and the March of Dimes and to S.S.S. from the NHLBI (HL09163). T.T. was supported by grants from the Delaware Valley and Brandywine Valley Hemophilia Foundations. V.L.S. is supported by a grant from the NIMH (1K08MH/NS63886-01). V.L.S. is a Charles A. Dana and CITP fellow. Y.F. is a Charles A. King postdoctoral fellow.

REFERENCES

1. Poussaint, T.Y., Fox, J.W., Dobyns, W.B., Radtke, R., Scheffer, I.E., Berkovic, S.F., Barnes, P.D., Huttenlocher, P.R. and Walsh, C.A. (2000) Periventricular nodular heterotopia in patients with filamin-1 gene mutations: neuroimaging findings. *Pediatr. Radiol.*, **30**, 748–755.
2. Andermann, E., Andermann, F., Dubeau, F. and Lee, N. (1994) Periventricular nodular heterotopia. *Neurology*, **44**, 581–582.
3. Huttenlocher, P.R., Taravath, S. and Mojtahedi, S. (1994) Periventricular heterotopia and epilepsy. *Neurology*, **44**, 51–55.
4. Fox, J.W., Lamperti, E.D., Eksioglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A. *et al.* (1998) Mutations in Filamin A prevent migration of cerebral cortical neurons in human periventricular heterotopia. *Neuron*, **21**, 1315–1325.
5. Fox, J.W. and Walsh, C.A. (1999) Periventricular heterotopia and the genetics of neuronal migration in the cerebral cortex. *Am. J. Hum. Genet.*, **65**, 19–24.
6. Sheen, V.L., Dixon, P.H., Fox, J.W., Hong, S.E., Kinton, L., Sisodiya, S.M., Duncan, J.S., Dubeau, F., Scheffer, I.E., Schachter, S.C. *et al.* (2001) Mutations in the X-linked Filamin A gene cause periventricular nodular heterotopia in males as well as in females. *Hum. Mol. Genet.*, **10**, 1775–1783.
7. Moro, F., Carrozzo, R., Veggiotti, P., Tortorella, G., Toniolo, D., Volzone, A. and Guerrini, R. (2002) Familial periventricular heterotopia: Missense and distal truncating mutations of the FLNA gene. *Neurology*, **58**, 916–921.
8. Gorlin, J.B., Yamin, R., Egan, S., Stewart, M., Stossel, T.P., Kwiatkowski, D.J. and Hartwig, J.H. (1990) Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J. Cell Biol.*, **111**, 1089–1105.
9. Stossel, T.P., Condeelis, J., Cooley, L., Hartwig, J.H., Noegel, A., Schleicher, M. and Shapiro, S.S. (2001) Filamins as integrators of cell mechanics and signalling. *Nat. Rev. Mol. Cell. Biol.*, **2**, 138–145.
10. Takafuta, T., Wu, G., Murphy, G.F. and Shapiro, S.S. (1998) Human beta-filamin is a new protein that interacts with the cytoplasmic tail of glycoprotein Ibalpha. *J. Biol. Chem.*, **273**, 17531–17538.
11. Wakeman, J.A., Walsh, J. and Andrews, P.W. (1998) Human Wnt-13 is developmentally regulated during the differentiation of NTERA-2 pluripotent human embryonal carcinoma cells. *Oncogene*, **17**, 179–186.
12. Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C. and Fogh, J. (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. *Lab. Invest.*, **50**, 147–162.
13. Andrews, P.W. (1988) Human teratocarcinomas. *Biochim. Biophys. Acta*, **948**, 17–36.
14. Zhang, W., Han, S.W., McKeel, D.W., Goate, A. and Wu, J.Y. (1998) Interaction of presenilins with the filamin family of actin-binding proteins. *J. Neurosci.*, **18**, 914–922.
15. Ozanne, D.M., Brady, M.E., Cook, S., Gaughan, L., Neal, D.E. and Robson, C.N. (2000) Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. *Mol. Endocrinol.*, **14**, 1618–1626.
16. Ott, I., Fischer, E.G., Miyagi, Y., Mueller, B.M. and Ruf, W. (1998) A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. *J. Cell Biol.*, **140**, 1241–1253.
17. Stahlhut, M. and van Deurs, B. (2000) Identification of Filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1-associated membrane domains by the actin cytoskeleton. *Mol. Biol. Cell*, **11**, 325–337.
18. Leonardi, A., Ellinger-Ziegelbauer, H., Franzoso, G., Brown, K. and Siebenlist, U. (2000) Physical and functional interaction of filamin (actin-binding protein-280) and tumor necrosis factor receptor-associated factor 2. *J. Biol. Chem.*, **275**, 271–278.
19. Nagano, T., Hatanaka, Y., Kubota, D., Yagi, H., Murakami, F. and Sato, M. (2000) FILIP: a novel filamin-interacting protein that potentially regulates cell migration in the developing cerebral cortex. *Soc. Neurosci. Abstr.*, **26**, 110.
20. Santi, M.R. and Golden, J.A. (2001) Periventricular heterotopia may result from radial glial fiber disruption. *J. Neuropathol. Exp. Neurol.*, **60**, 856–862.
21. Weihing, R.R. (1988) Actin-binding and dimerization domains of HeLa cell filamin. *Biochemistry*, **27**, 1865–1869.
22. van der Flier, A., Kuikman, I., Kramer, D., Geerts, D., Kreft, M., Takafuta, T., Shapiro, S.S. and Sonnenberg, A. (2002) Different splice variants of filamin-B affect myogenesis, subcellular distribution, and determine binding to integrin [beta] subunits. *J. Cell Biol.*, **156**, 361–376.
23. Bellanger, J.M., Astier, C., Sardet, C., Ohta, Y., Stossel, T.P. and Debant, A. (2000) The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nat. Cell. Biol.*, **2**, 888–892.
24. Bellanger, J.M., Zugasti, O., Lazaro, J.B., Diriong, S., Lamb, N., Sardet, C. and Debant, A. (1998) Role of the multifunctional Trio protein in the control of the Rac1 and RhoA gtpase signaling pathways. *C. R. Seances Soc. Biol. Fil.*, **192**, 367–374.
25. Marti, A., Luo, Z., Cunningham, C., Ohta, Y., Hartwig, J., Stossel, T.P., Kyriakis, J.M. and Avruch, J. (1997) Actin-binding protein-280 binds the stress-activated protein kinase (SAPK) activator SEK-1 and is required for tumor necrosis factor-alpha activation of SAPK in melanoma cells. *J. Biol. Chem.*, **272**, 2620–2628.
26. Takafuta, T., Jiang, D., Ruf, W. and Shapiro, S.S. (1999) Expression and function of filamin homologs in a Filamin A deficient melanoma cell line. *Mol. Biol. Cell*, **10**, 154a.
27. Sheen, V.L. and Macklis, J.D. (1994) Apoptotic mechanisms in targeted neuronal cell death by chromophore-activated photolysis. *Exp. Neurol.*, **130**, 67–81.
28. Wang, Y., Sheen, V.L. and Macklis, J.D. (1998) Cortical interneurons upregulate neurotrophins in vivo in response to targeted apoptotic degeneration of neighboring pyramidal neurons. *Exp. Neurol.*, **154**, 389–402.
29. Berger, U.V. and Hediger, M.A. (2001) Differential distribution of the glutamate transporters GLT-1 and GLAST in tanycytes of the third ventricle. *J. Comp. Neurol.*, **433**, 101–114.
30. Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.
31. Feng, Y., Olson, E.C., Stukenberg, P.T., Flanagan, L.A., Kirschner, M.W. and Walsh, C.A. (2000) LIS1 regulates CNS lamination by interacting with mNudE, a central component of the centrosome. *Neuron*, **28**, 665–679.