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Serine 732 Phosphorylation of FAK by Cdk5 Is Important for Microtubule Organization, Nuclear Movement, and Neuronal Migration

Zhigang Xie,[1] Kamon Sanada,[1,4] Benjamin Adam Samuels,[1,2,4] Heather Shih,[1] and Li-Huei Tsai[1,2,3,*]
[1]Department of Pathology
Harvard Medical School
[2]Program in Neuroscience
Harvard University
[3]Howard Hughes Medical Institute
200 Longwood Avenue
Boston, Massachusetts 02115

Summary

The serine/threonine kinase Cdk5 plays an essential role in neuronal positioning during corticogenesis, but the underlying mechanisms are unknown. In nonneuronal cells, the tyrosine kinase FAK is a major regulator of cell motility through focal adhesions. It is unclear whether FAK plays a role in brain development. Here, we show that FAK phosphorylation by Cdk5 at S732 is important for microtubule organization, nuclear movement, and neuronal migration. In cultured neurons, S732-phosphorylated FAK is enriched along a centrosome-associated microtubule fork that abuts the nucleus. Overexpression of the nonphosphorylatable mutant FAK S732A results in disorganization of the microtubule fork and impairment of nuclear movement in vitro, and neuronal positioning defects in vivo. These observations are reminiscent of what is seen in the Cdk5-deficient mice. Taken together, these results suggest that Cdk5 phosphorylation of FAK is critical for neuronal migration through regulation of a microtubule fork important for nuclear translocation.

Introduction

Formation of the cerebral cortex occurs in a highly organized fashion. Individual layers are formed by successive waves of postmitotic neurons that migrate radially from the ventricular zone toward the pia mater. Corticogenesis begins with the formation of a preplate by the first wave of postmitotic neurons. The second wave splits the preplate into a superficial marginal zone and a deeper subplate, forming the cortical plate in between. The cortical plate is then expanded in an "inside-out" manner, with earlier-born neurons forming deeper layers and later-born neurons migrating past their predecessors to form more superficial layers (Hatten, 2002; Nadarajah and Parnavelas, 2002; Ross and Walsh, 2001).

Migration of a neuron consists of three sequential steps. First, there is extension of a leading process, which is followed by nucleokinesis, the act of nuclear translocation in the leading process. The final step is retraction of the trailing process. It is believed that RhoGTPases play a role in regulating leading process extension and that nucleokinesis is critically dependent on the microtubule network (Lambert de Rouvroit and Goffinet, 2001; Luo, 2000; Walsh and Goffinet, 2000). Detailed molecular mechanisms underlying the regulation of these steps, however, have not been elucidated.

Analysis of loss-of-function mouse models and human disease genes has uncovered several molecules that regulate corticogenesis. These include the actin crosslinking protein filamin 1 (Fox et al., 1998); proteins that regulate microtubule organization and dynamics, such as Lis1 (Reiner et al., 1993) and Doublecortin (Gleeson et al., 1998); astrotactin, which functions in neuron-glia interactions (Adams et al., 2002; Zheng et al., 1996); integrin receptors (Anton et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001); proteins in the reelin pathway, such as Reelin (D'Arcangelo et al., 1995), VLDLR, ApoER2 (D'Arcangelo et al., 1999; Trommsdorff et al., 1999), and mDab1 (Howell et al., 1997; Sheldon et al., 1997); the transcription factors Brn-1 and Brn-2 (McEvilly et al., 2002; Sugitani et al., 2002); and the serine/threonine kinase Cdk5 (Ohshima et al., 1996) and its essential activators p35 and p39 (Chae et al., 1997; Ko et al., 2001).

The highest level of Cdk5 activity is present in postmitotic neurons of the central nervous system, overlapping with the expression pattern of its activators p35 and p39. Cdk5-deficient mice, which die around birth, show an inverted lamination of neurons in the cerebral cortex. While splitting of the preplate is preserved, the normal inside-out gradient of corticogenesis is supplanted by an outside-in order in which later-born neurons fail to migrate past their predecessors and accumulate in the deeper layers. Double knockouts of p35 and p39 show an identical phenotype to the Cdk5 deficient mice, suggesting that loss of Cdk5 kinase activity is responsible for neuronal positioning defects in these mutant mice (Gupta et al., 2002).

As Cdk5 kinase activity is required for proper development of the neocortex, the identification of Cdk5 substrates should elucidate the underlying mechanisms of how this kinase regulates corticogenesis. A diverse array of proteins phosphorylated by Cdk5 has been discovered (Dhavan and Tsai, 2001), among which Nudel, mDab1, and Pak1 are potential substrates during neocortical development (Niethammer et al., 2000; Sasaki et al., 2000; Keshvara et al., 2002; Nikolic et al., 1998; Rashid et al., 2001). However, a direct role for any of these proteins in mediating the function of Cdk5 during neuronal migration has not been established, and the mechanisms underlying the role of Cdk5 in the developing neocortex remain undiscovered.

Focal adhesion kinase (FAK) was originally identified as a tyrosine kinase localized to focal adhesions, which are multiprotein structures that link the extracellular matrix to the actin cytoskeleton through integrin receptors (Hanks et al., 1992; Schaller et al., 1992). FAK has been shown to be critical for cell migration (Parsons et al., 2000; Schaller, 2001), although the underlying mechanism is unclear. The current understanding is that FAK modulates the dynamics of the actin cytoskeleton at

*Correspondence: li-huei_tsai@hms.harvard.edu
[4]These authors contributed equally to this work.

focal adhesions to promote cell migration (Parsons et al., 2000; Schaller, 2001). In mammals, FAK is expressed at the highest level in brain and is enriched in neurons (Andre and Becker-Andre, 1993; Burgaya et al., 1995; Grant et al., 1995; Hanks et al., 1992). Neurons do not possess typical focal adhesion structures seen in nonneuronal cells, though neurites and growth cones might contain protein assemblies analogous to focal adhesions (Contestabile et al., 2003; Renaudin et al., 1999; Stevens et al., 1996). Since FAK null mice die prior to the majority of brain development (Ilic et al., 1995), it remains undetermined whether FAK plays a role in the development of the nervous system, and if FAK functions in neurons through mechanisms other than modulation of focal adhesion dynamics.

In the present study, we identify FAK as a physiological substrate of Cdk5 during neocortical development. We present evidence that S732 phosphorylation of FAK is Cdk5-dependent and critical for organization not of the actin cytoskeleton but, rather, a small network of microtubules that partially encompass the nucleus. We demonstrate that a nonphosphorylatable FAK S732A mutant disorganizes this microtubule network and impairs both nuclear movement in vitro and neuronal migration in vivo.

Results

Cdk5 Phosphorylates FAK at S732 In Vitro

To find substrates important for the development of the brain, we searched for Cdk5 consensus sequences in proteins involved in cell migration. Cdk5 phosphorylates serines or threonines upstream of a proline residue but prefers sites that also contain a basic residue in the +3 position. Mouse FAK contains several proline-directed serines and threonines and one site (S732), which is optimal for Cdk5 phosphorylation (SPQH). To investigate if Cdk5 phosphorylates FAK, different domains were fused to GST, expressed in bacteria, and purified (Figure 1A). GST-FAK-3, which encompasses most of the proline-rich domain, was the only fragment phosphorylated in vitro by Cdk5 (Figure 1B). This fragment contains the optimal site S732, as well as a potential site at S722. Mutagenesis of these sites to nonphosphorylatable alanine residues shows that S732 is the only site phosphorylated by Cdk5, as phosphorylation of the S732A fragment is undetectable, while the S722A fragment is still phosphorylated by Cdk5 (Figure 1C). The residues composing the consensus site for Cdk5 phosphorylation at S732 are conserved throughout vertebrates (Figure 1D), suggesting that they may be important for the function of FAK.

Cdk5 Phosphorylates FAK at S732 In Vivo

To analyze phosphorylation of FAK at S732 in vivo, we made a phosphorylation state-specific antibody. The phospho-S732 antibody (pS732) recognizes wild-type and S722A-GST-FAK-3 fragments that have been phosphorylated, but not S732A-GST-FAK or nonphosphorylated fragments (Figure 2A), indicating that the antibody is specific to S732-phosphorylated FAK.

In embryonic mouse brain lysate, the pS732 antibody recognizes a band corresponding to the size of FAK (Figure 2B, lane 1). When the lysate is precleared with pan-FAK antibodies (Figure 2B, lane 2), the intensity of this band is diminished. Furthermore, pS732 recognizes immunoprecipitates made with pan-FAK antibodies (Figure 2B, lane 4). This data indicates that the pS732 antibody recognizes a form of FAK present in embryonic mouse brains.

To determine if Cdk5 phosphorylates S732 in vivo, we probed brain lysates from mice lacking Cdk5 or its activators. Lack of p35 resulted in a decrease of S732 phosphorylation at several developmental stages (Figure 2C). Lack of Cdk5 or both activators abolishes phosphorylation of FAK at S732 (Figure 2D), while S722 phosphorylation is unaffected (pS722, Biosource). This data indicates that S732 phosphorylation is dependent on Cdk5 activity at different developmental stages in vivo. Furthermore, FAK S732 phosphorylation is markedly reduced in adult brain lysates (Figure 2C), suggesting a developmental role for Cdk5 phosphorylation of FAK.

We also checked for differences in FAK activity in the absence of S732 phosphorylation. Both tyrosine 397 and overall FAK tyrosine phosphorylation are commonly used as readouts of activity (Parsons et al., 2000; Schaller, 2001). Cdk5-deficient brains from several development stages do not show a consistent difference in either readout relative to wild-type littermates, suggesting that phosphorylation at S732 does not impact directly on the catalytic activity of FAK (data not shown).

Cdk5 Phosphorylated FAK Is Present in Postmitotic Neurons of the Developing Neocortex

The pS732 antibody was next used to determine localization in brain sections of mouse embryos. The antibody recognizes cells from the intermediate zone to the marginal zone in a section from an E16 mouse (Figure 3A). The postmitotic neuronal marker Hu shows a similar staining pattern, suggesting that Cdk5 phosphorylated FAK is mostly localized to neurons. When the pS732 antibody is preabsorbed with the phosphopeptide used as antigen (Figure 3B), or sections are pretreated with phosphatase (Figure 3C), the staining is abolished. Most of the staining is also eliminated in sections from Cdk5 null embryonic brains, though a slight nonspecific background remains (Figure 3D). This data suggests that Cdk5-dependent S732-phosphorylated FAK is present in postmitotic neurons throughout most of the developing neocortex.

S732-Phosphorylated FAK Localizes to a Perinuclear Region around the Centrosome and to Microtubules

We next assessed the subcellular localization of Cdk5-phosphorylated FAK by immunostaining neocortical cultures. Z series images taken with a confocal microscope show phospho-FAK decorates bundles of microtubules on top of roughly half of the nucleus (Figure 4A, Z2, white arrows). This network of microtubules consists of a structure with two or more branches of thick β III tubulin-positive bundles abutting the nucleus that converge at a perinuclear site, giving the appearance of a fork. This pS732 staining pattern is not observed in cultures that were made from Cdk5-deficient mice (see

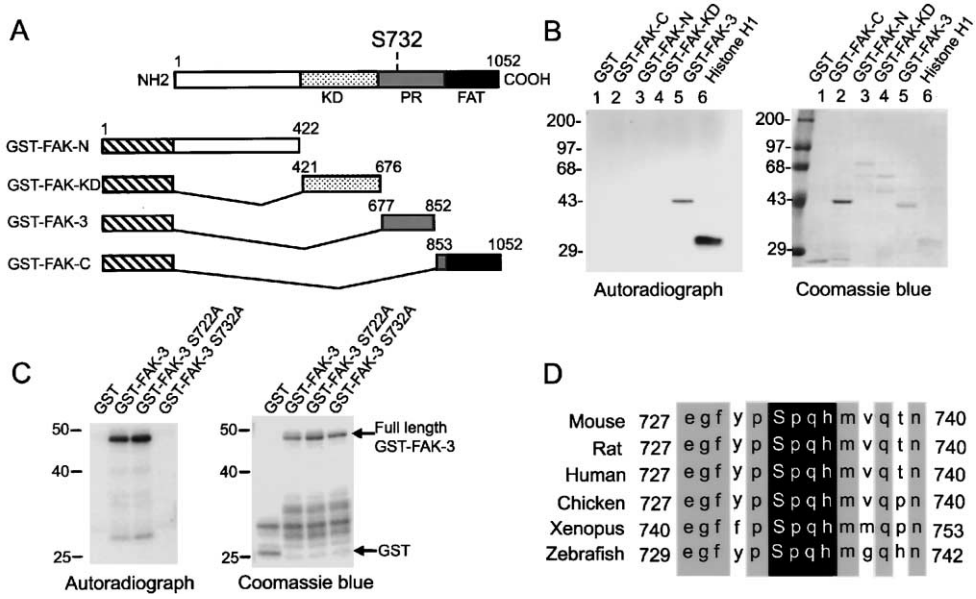

Figure 1. Cdk5 Phosphorylates FAK at S732 In Vitro

(A) GST-FAK domain fragments are shown. Abbreviations: KD, kinase domain; PR, proline-rich domain; FAT, focal adhesion targeting domain.
(B) In vitro kinase assays were performed using p35/Cdk5 produced in bacculovirus. Histone H1 was used as a positive control.
(C) GST-FAK-3 fragments with the S732A or S722A mutation subjected to in vitro phosphorylation by purified GST-p25/GST-Cdk5.
(D) The Cdk5 consensus site (SPQH) is conserved.

Supplemental Figure S1A, Z2 online at http://www.cell.com/cgi/content/full/114/4/469/DC1) or that were CIP treated or preabsorbed by the antigen peptide (data not shown), even though there is a slight background of dots in the nucleus seen with the pS732 antibody (Figure 4A, Z1; Supplemental Figure S1A, Z1). In a Z section, α-tubulin colocalization confirms that Cdk5-phosphorylated FAK adorns branches of a microtubule fork (Figure 4B, white arrow).

We also used transparent 3D reconstruction, which merges several Z sections but allows for visualization of fluorescent intensity throughout the volume of the cell, to show that the branches of the fork (white arrows) and their perinuclear convergence point (white arrowhead) are some of the most prominent microtubule structures in the cell body (Figure 4C). We next used nontransparent 3D reconstruction to merge several Z sections and study the position of the microtubule fork relative to the nucleus (Figure 4D). With nontransparent 3D reconstruction, only the fluorescence closest to the surface of the cell is visible, and deeper staining is blocked. Rotation of the nontransparent 3D reconstruction shows that the microtubule fork abuts the nucleus (Figure 4D2 white arrows).

We also noticed that phospho-S732 FAK is distributed in a punctate perinuclear fashion (Figure 4A, Z1). In a single Z section, costaining of pS732 with γ-tubulin shows that phospho-FAK is present at the centrosome, where the microtubule organizing center (MTOC) is hosted (Figure 4E). Costaining of β III tubulin with γ-tubulin also indicates that the branches of the microtubule fork converge at the centrosome (data not shown).

Most studies in nonneuronal cells indicate a functional role for FAK in the regulation of signaling between the extracellular matrix and the actin cytoskeleton at focal adhesion structures. In neurons, protein assemblies analogous to focal adhesions of nonneuronal cells have been described in neurites and growth cones where FAK colocalizes with both vinculin and F-actin (Contestabile et al., 2003; Renaudin et al., 1999; Stevens et al., 1996). The majority of phospho-S732 FAK, however, is distributed in a perinuclear fashion and along the microtubule fork (Figure 4F) and does not overlap with these focal adhesion-like structures. Furthermore, a transparent 3D reconstruction shows that phospho-FAK does not display robust colocalization with F-actin (Figure 4F).

We also performed immunostaining with an antibody against pan-FAK, which displays a broader staining pattern (Andre and Becker-Andre, 1993; Burgaya et al., 1995; Grant et al., 1995) than phospho-FAK. Different Z sections, however, demonstrate that pan-FAK stains a perinuclear region (Figure 4G, Z1) and the microtubule fork (Figure 4G, Z2, white arrows). Thus, a population of FAK in neurons colocalizes with the microtubule fork.

To further characterize the microtubule fork, we found that Lis1, a protein critical for proper corticogenesis (Reiner et al., 1993), localizes to the thick β III tubulin-positive branches (Figure 4H, Z2). In addition, we found that the Lis1 binding partner Nudel and the retrograde motor protein Dynein adorn the microtubule fork branches (Supplemental Figures S1C, Z2 and S1D, Z2). Lis1, Nudel, and Dynein are also localized to the centrosome (Figure 4H, Z1 and Supplemental Figures S1C, Z1 and S1D, Z1) (Niethammer et al., 2000; Sasaki et al., 2000).

To confirm the immunostaining, which suggests a role for phospho-FAK at microtubules, we found that phospho-FAK is present in a tubulin preparation from bovine brain enriched for microtubule-associated proteins (Cy-

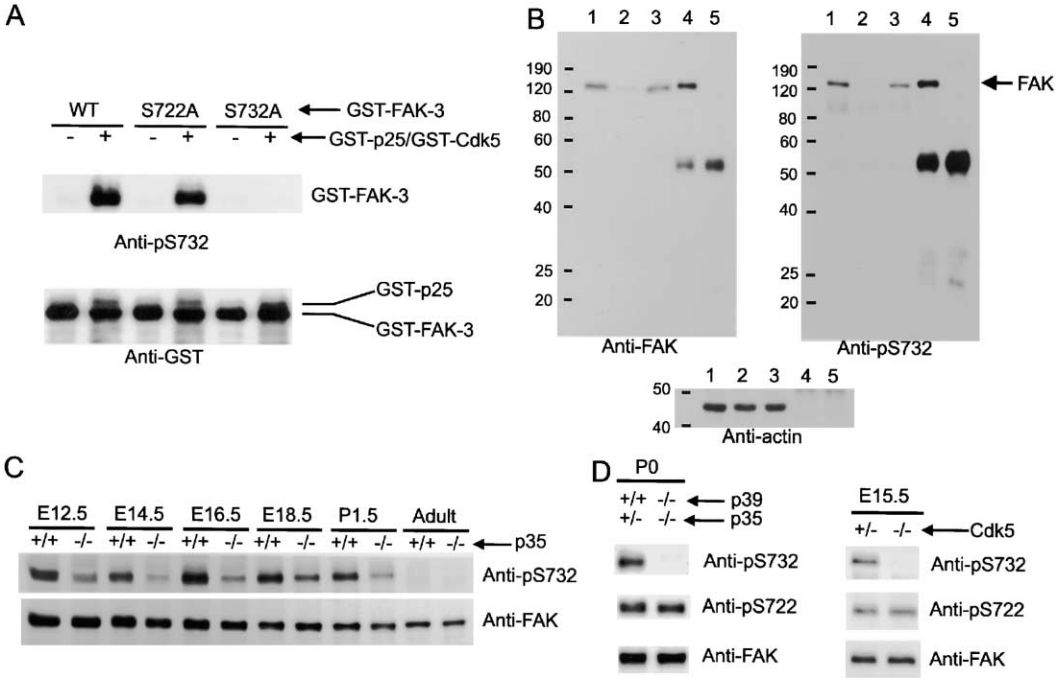

Figure 2. Cdk5 Phosphorylates FAK at S732 In Vivo

(A) GST-FAK-3, S722A-GST-FAK-3, and S732A-GST-FAK-3 were phosphorylated in vitro by GST-p25/GST-Cdk5. The reaction mixture was immunoblotted with the phospho-S732 FAK antibody.

(B) E18.5 mouse brain lysates (lane 1, 30 μg), precleared by FAK (lane 2, 30 μg) or GST antibodies (lane 3, 30 μg), and FAK (lane 4) or GST (lane 5) immunoprecipitates (from 200 μg lysates) were analyzed by anti-pS732 Western blot. Actin was used as a loading control.

(C) Brain lysates from wild-type or p35-deficient mice of different ages were analyzed by anti-pS732 immunoblot.

(D) Brain lysates from Cdk5-deficient mice or mice lacking both p35 and p39 were analyzed by anti-pS732 and anti-pS722 Western blot.

toskeleton, Inc.) and in the microtubule pellet of COS7 cells transfected with Cdk5 and p35, but not the microtubule supernatant (Figure 4I). Lis1, a known regulator of microtubule dynamics (Sapir et al., 1997), is found in both the microtubule supernatant and pellet from transfected COS7 cells (Figure 4I), further suggesting that phospho-FAK localizes specifically to microtubules.

Overexpression of Nonphosphorylatable FAK Causes Disorganization of the Microtubule Fork

The localization of phospho-FAK at the MTOC and to the microtubule fork led us to hypothesize that FAK phosphorylation by Cdk5 at S732 is important for organization of microtubules. We thus transfected neocortical cultures with GFP, GFP-FAK, or GFP-FAK-S732A. Consistent with the immunocytochemistry, GFP-FAK labels a fork structure on top of the nucleus that is β III tubulin-positive (Figure 5A, left, Z2, white arrows). Expression of GFP has no effect on the robust branches of the microtubule fork (data not shown). In neurons transfected with GFP-FAK-S732A, however, a prominent microtubule fork lying above the nucleus is not observed (Figure 5A, right, Z2).

To further understand this apparent disorganization of the microtubule fork, we analyzed transparent 3D reconstructed images (Figure 5B). A clear network of robust microtubule branches on top of the nucleus was observed in 65.1% ± 4.5% (mean ± SEM; $n = 4$) of neurons expressing GFP-FAK (Figure 5C). A prominent microtubule fork was observed in significantly fewer neurons expressing GFP-FAK-S732A (18.1% ± 2.5%, $p < 0.001$ relative to GFP-FAK; $n = 4$). Unlike the GFP-FAK expressing cell, which has two prominent branches where most of the β III tubulin staining is concentrated (Figure 5B, top), the GFP-FAK-S732A-expressing neuron phenotype ranges from small or no discernible fork-like structures to thin bundles and diffuse β III tubulin staining (Figure 5B, bottom three panels). This data suggests that FAK phosphorylation by Cdk5 at S732 plays an important role in the organization of the microtubule fork.

We next looked at the effects of GFP-FAK-S732A overexpression on endogenous phospho-S732 FAK. Cultured neurons expressing GFP-FAK-S732A demonstrate much less pS732 staining in the perinuclear region relative to nearby neurons without detectable GFP signal (Figure 5D). This effect is specific to phospho-FAK, as Cdk5 phosphorylation of Nudel (Patzke et al., 2003) is unaffected in neurons overexpressing GFP-FAK-S732A (Supplemental Figure S2). This data is consistent with FAK-S732A-GFP replacing the endogenous FAK (Supplemental Figure S2), thus eliminating phospho-FAK from the centrosome and the microtubule fork rather than exerting its effects through a dominant neomorphic activity. Furthermore, while GFP-FAK and GFP-FAK-S732A colocalize with vinculin in neurites and F-actin in growth cones, these structures seem unaffected by S732A overexpression (Figure 5E).

To further investigate the role of S732 phosphorylation, we used transparent 3D reconstructed images to assess the prominence of the fork in primary neocortical cultures made from mice deficient in Cdk5, where S732

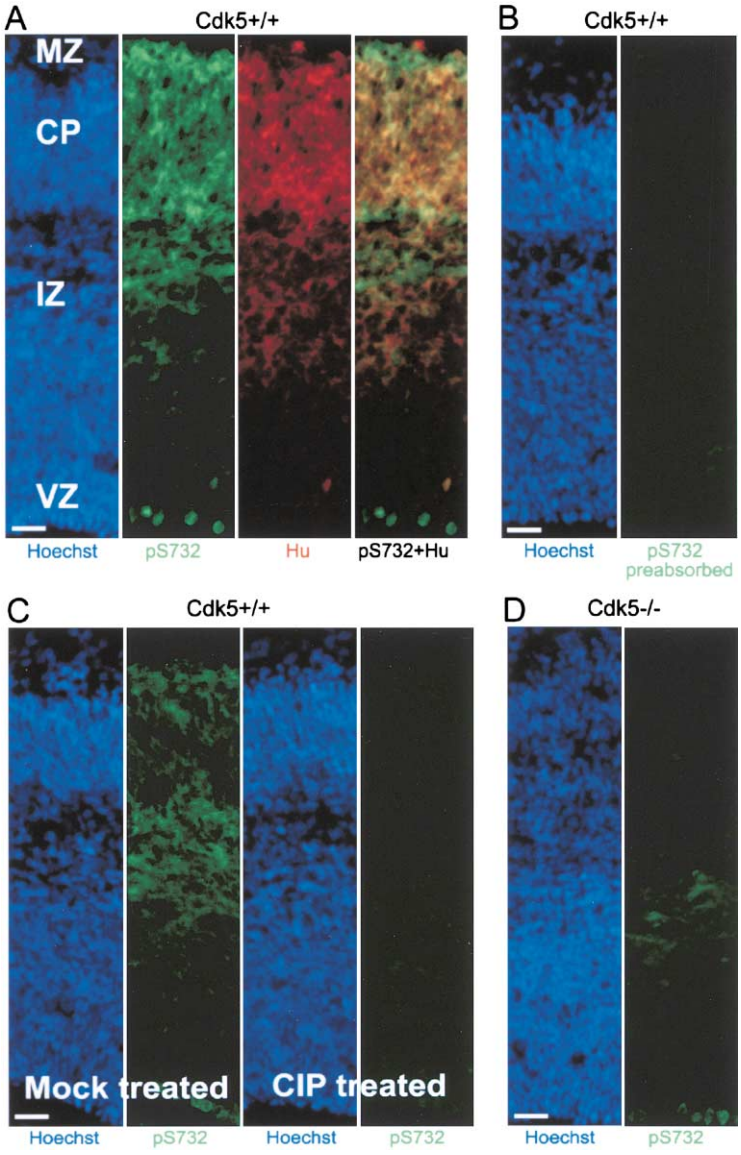

Figure 3. S732 Phosphorylated FAK Is Present in Postmitotic Neurons of the Developing Neocortex

(A) Coronal brain cryosections from an E16 wild-type mouse were costained with the pS732 antibody preabsorbed by a nonphospho-S732 peptide and an antibody against Hu, a marker for postmitotic neurons. The layers of the neocortex were visualized by nuclear staining with Hoechst 33258. Abbreviations: MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

(B) The brain cryosections were stained with the pS732 antibody preabsorbed by the phosphopeptide used as antigen.

(C) The brain cryosections were treated with H_2O or calf intestinal phosphatase (CIP) and then stained with the pS732 antibody preabsorbed with a nonphospho-S732 peptide.

(D) Brain coronal cryosections from a Cdk5-deficient littermate were stained with the pS732 antibody preabsorbed with a nonphospho-S732 peptide. Scale bars, 20 μm.

phosphorylation is undetectable. Parallel neocortical cultures from littermates of Cdk5 heterozygote crossings were analyzed at day in vitro (DIV) 2. While the fork branches are thick in neurons of wild-type littermates (Figure 5F, left), there is a general disorganization of microtubules demonstrated by diffuse β III tubulin staining above the nucleus in the Cdk5-deficient mice (Figure 5F, right). Any branches are much thinner than what is seen in the fork structure of wild-type neurons. As the microtubule fork is disrupted in both the absence of Cdk5 and with the overexpression of a mutant nonphosphorylatable FAK, Cdk5 phosphorylation of FAK at S732 is likely critical for organization of the microtubule fork.

In Utero Electroporation of FAK-S732A into the Embryonic Cerebral Wall Results in Neuronal Positioning Defects In Vivo

We next tested to see if Cdk5 phosphorylation of FAK at S732 had any effects on neuronal positioning in vivo by expressing the GFP-FAK-S732A mutant in the developing cortex with an in utero electroporation technique that allows for cells in the ventricular zone of early stage embryos to be transfected (Figure 6A) (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Since we used a GFP tag, migration of transfected neurons can be followed by determining the position of fluorescent cells at different time points following the electroporation. Using plasmids encoding GFP or GFP-FAK as controls, we electroporated E14 mice and assessed the distribution of fluorescent cells 3 days later (Figure 6B). Due to the difficulty in distinguishing neurons from radial glial cells in the ventricular and subventricular zone, only fluorescent cells that had already migrated out of these zones were quantified. In embryos that had been electroporated with GFP or GFP-FAK, about 50% (45.7% ± 3.2% and 51.0% ± 2.1%, mean ± SEM, for GFP and GFP-FAK respectively) of the fluorescent cells were found in the superficial cortical plate (Figure 6C). In embryos electroporated with GFP-FAK-S732A, most transfected cells that had migrated out of the ventricular zone were not found in the superficial cortical plate (only 10.7% ± 4.3%, $p < 0.001$ relative to GFP and GFP-FAK;

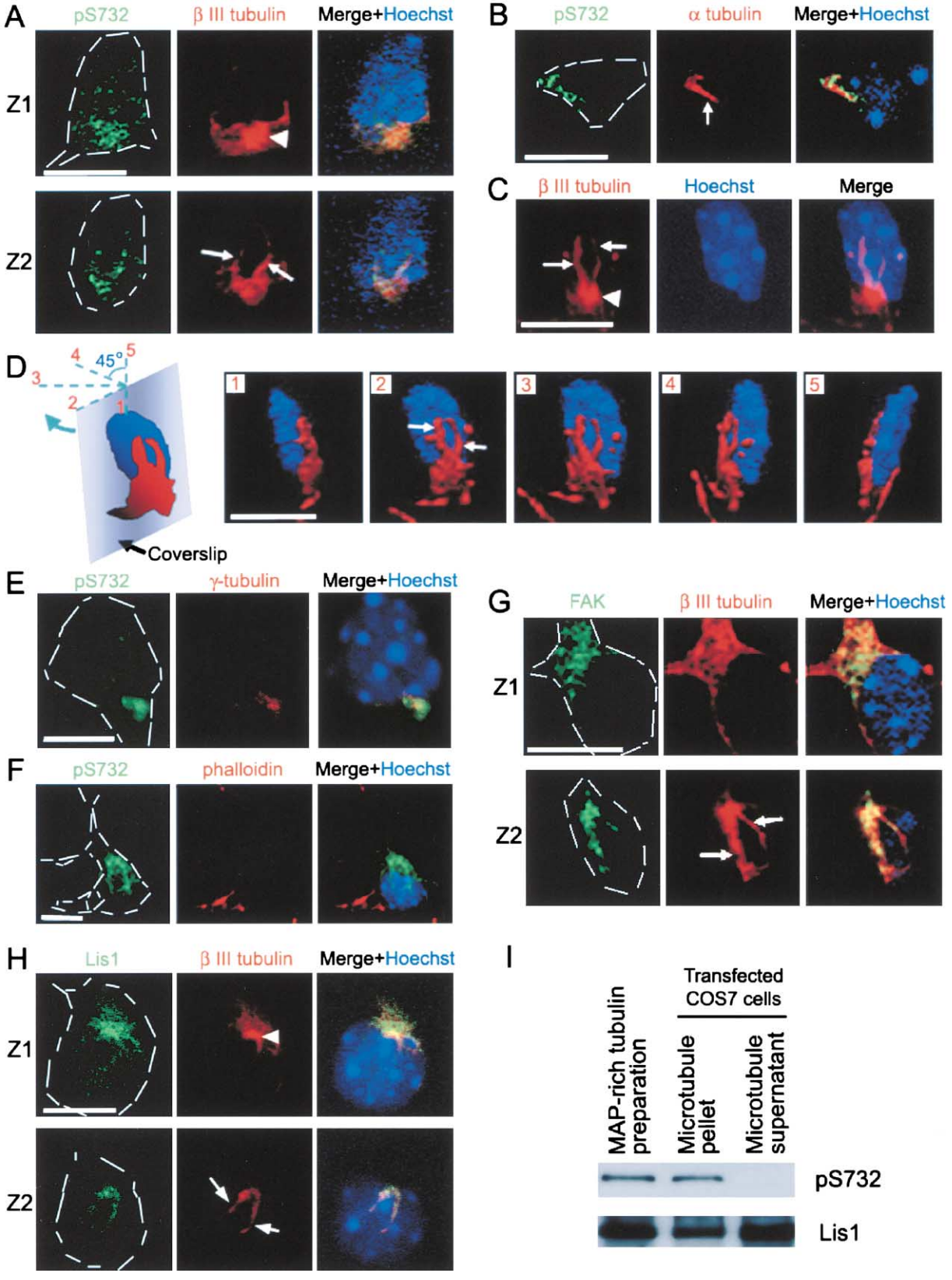

Figure 4. Enrichment of S732-Phosphorylated FAK on a Microtubule Fork

(A–F) Neocortical neurons at DIV2 were immunostained with indicated antibodies and Hoechst 33258 and analyzed by confocal microscopy. (A and B) A microtubule fork (recognized by β III tubulin or α-tubulin) is shown by Z sections (Z1 is closer to the culture substratum than Z2). The pS732 antibody labels both the converging point (Z1 of [A]) and the branches (Z2 of [A] and [B]) of the fork. (C) A transparent 3D

n = 3) but, instead, were observed in the intermediate zone and lower cortical plate. Thus, expression of the mutant GFP-FAK-S732A results in neuronal positioning defects in the developing neocortex.

An Altered Nuclear Morphology Is Seen in Neocortical Cells Expressing GFP-FAK-S732A or Lacking Cdk5

Nuclei of neurons transfected with GFP-FAK consistently had elongated, oval shapes, while GFP-FAK-S732A transfected cells often had more rounded nuclei. To quantify this nuclear morphology difference, we defined the nuclear diameter in the direction of the leading process as nuclear length and a perpendicular direction as nuclear width, and assessed the ratio of nuclear length relative to width. This ratio is significantly decreased in neurons expressing mutant FAK relative to FAK (Figure 6D; $p < 0.0001$; $n = 31$). We also looked at nuclear morphology in brain sections from Cdk5-deficient embryos (Figure 6E). Quantification of the most elongated nuclei in the cortical plate and intermediate zone revealed that Cdk5-deficient nuclei are significantly more rounded than those of wild-type littermates (Figure 6E; $p < 0.0001$; $n = 25$ for cortical plate, and $p < 0.0001$; $n = 25$ for intermediate zone). This data suggests that in utero electroporation of S732A-FAK into the embryonic cerebral wall recapitulates in vivo a nuclear morphology phenotype seen in Cdk5-deficient neurons and that there may be an association between neuronal positioning defects and more rounded nuclei.

Overexpression of GFP-FAK-S732A Impairs Nuclear Movement in Cultured Neocortical Neurons

The potential association between neuronal positioning defects and nuclear morphology abnormalities seen in vivo led us to investigate if we could recapitulate the more rounded nuclei phenotype in vitro. To this end, we expressed GFP-FAK-S732A in cultured neocortical neurons. The neurons were transfected at DIV1 and nuclear morphology was assessed at DIV2. Plasmids encoding GFP-FAK or GFP with a membrane localization domain (Moriyoshi et al., 1996) were used as controls. The predominantly extranuclear localization of the expressed fluorescent proteins allowed for identification of nuclear morphology. We found that nuclei of neurons expressing GFP-FAK-S732A had a significantly decreased length/width ratio relative to GFP or GFP-FAK (data not shown and Figure 7B, black columns).

To further understand potential causes of the more rounded nuclei, we performed time-lapse imaging. In our culture system, a small percentage of neurons show visible movement of the nucleus in a time frame of 20–60 min. These neurons typically possess a thick process toward which the nucleus moves (Figure 7A). In neurons expressing GFP (Figure 7A, top; Supplemental Movie S1) or GFP-FAK (Figure 7A, middle; Supplemental Movie S2), the leading pole (proximal to the thick process) of the nucleus begins to move at a similar time and, ultimately, moves a similar distance as the trailing pole (distal to the thick process). The morphology of the nucleus remains relatively consistent throughout this entire movement, and no abnormalities are distinguishable. However, in neurons expressing GFP-FAK-S732A (Figure 7A, bottom; Supplemental Movies S3 and S4), the movement of the leading pole is often delayed relative to that of the trailing pole or barely observed throughout the recording period. This difference in movement between the two nuclear poles results in an altered morphology in which the nucleus transitions from an elongated, oval shape to a much more rounded form.

To quantify this change in nuclear morphology, we assessed the nuclear length to width ratio at different time points. The first time point was the beginning of the recording, which we described earlier as displaying a significant difference between GFP-FAK-S732A and GFP or GFP-FAK (Figure 7B, black columns). The second time point was defined as when the leading pole of the nucleus started moving or if it did not move during the entire recording period, at the end of recording. In neurons expressing GFP or GFP-FAK, the nuclear length/width ratio is not significantly different between the two time points. However, in neurons expressing GFP-FAK-S732A, the ratio is significantly decreased ($p < 0.05$, $n = 20$) at the second time point relative to the beginning of recording (Figure 7B). Therefore, at the start of imaging, the significant decrease seen in nuclear length/width of GFP-FAK-S732A-expressing neurons relative to GFP and GFP-FAK is likely a result of neurons that had already started to move and transition into a more rounded shape prior to the filming. These results suggest that S732-phosphorylated FAK plays a critical role in nuclear movement in cultured neocortical neurons.

Unphosphorylated FAK Accumulates at the Centrosome of Neurons

We noticed that the impairment in nuclear movement was accompanied by an accumulation of fluorescence at a perinuclear spot proximal to the thick process that was not observed in neurons expressing GFP-FAK. This fluorescence accumulation was observed in most cells with moving nuclei that expressed GFP-FAK-S732A (Figure 7A, bottom; Supplemental Movies S3 and S4). γ-tubulin staining of a neuron expressing GFP-FAK-S732A shows that the FAK accumulation occurs at the centrosome in the absence of S732 phosphorylation

reconstruction of the fork. (D) Nontransparent 3D reconstruction images of the fork from different angles. (E) The perinuclear pS732-positive spot is enriched for γ-tubulin. (F) A transparent 3D reconstruction shows F-actin (labeled by phalloidin) is enriched at growth cones but does not significantly overlap with pS732 staining.

(G) FAK C-20 labels both the perikarya (Z1) and the branches of the fork (Z2).

(H) Lis1 is at the converging point (Z1) and the branches (Z2) of the fork.

(I) S732-phosphorylated FAK is present in a MAP-rich brain tubulin prep and enriched in the microtubule pellet of COS7 cells overexpressing p35 and Cdk5. In all the panels, arrows and arrowheads indicate branches and the converging point of the microtubule fork, respectively. Cells are outlined with dashed white lines. Scale bars, 10 μm.

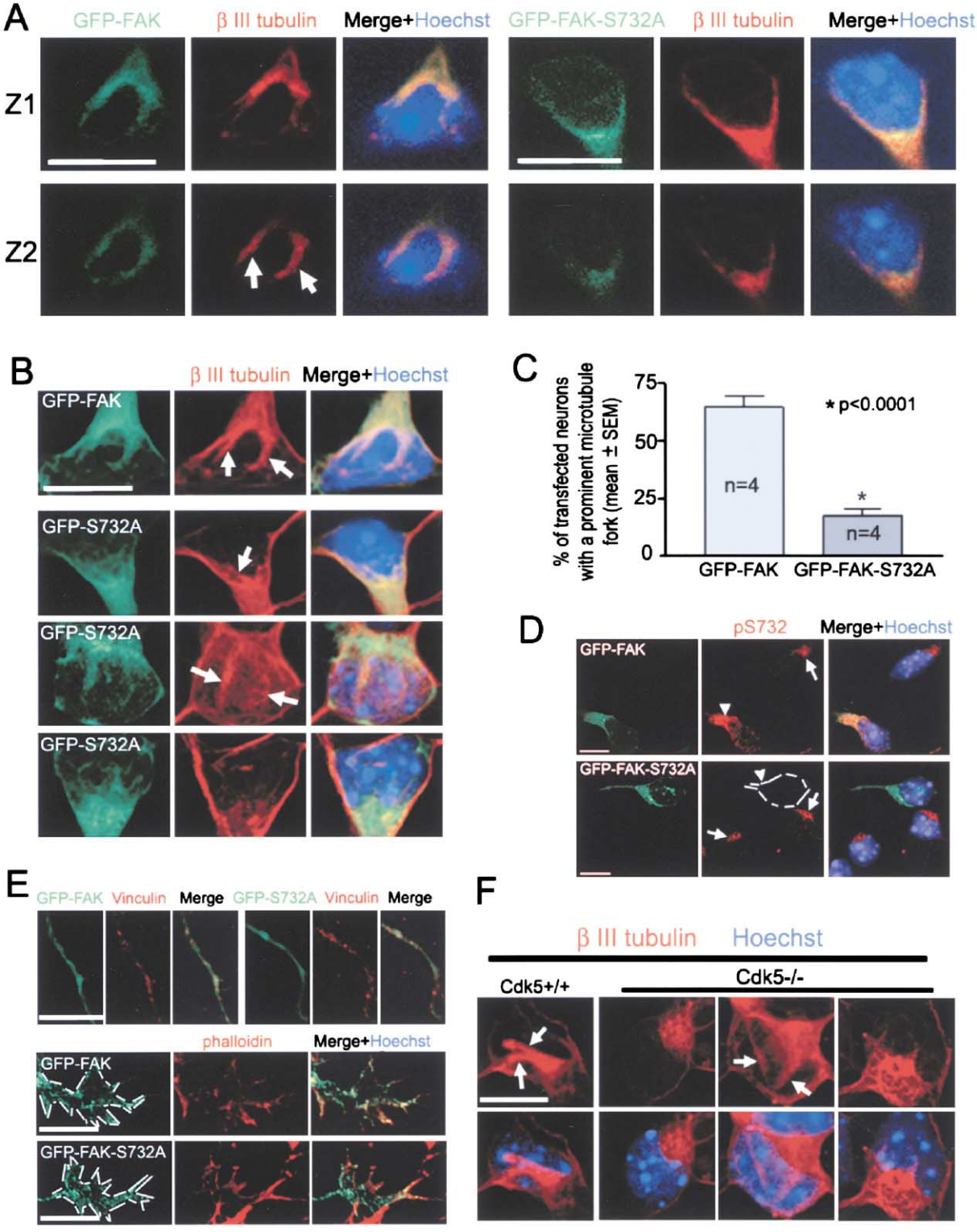

Figure 5. S732 Phosphorylation and the Organization of the Microtubule Fork

(A and B) Cocortical cultures were transfected with indicated plasmids at DIV1, fixed at DIV2, stained for β III tubulin and analyzed by confocal microscopy. The disorganized fork in neurons overexpressing GFP-FAK-S732A is shown in Z sections (A, right panels as compared to left panels) and transparent 3D reconstructed images (B, bottom three rows as compared to the top row). Arrows indicate branches of the fork in neurons expressing GFP-FAK or disorganized branches in neurons expressing GFP-FAK-S732A.

(C) Statistical analysis of the effect (one-way ANOVA, four independent experiments, 40–60 neurons quantified per experiment).

(D) Overexpression of GFP-FAK-S732A (bottom), but not GFP-FAK (top), reduces the perinuclear S732-phosphorylated FAK. Arrowheads indicate pS732-staining in transfected neurons. Arrows indicate pS732-staining in neurons without detectable fluorescent signal. The trans-

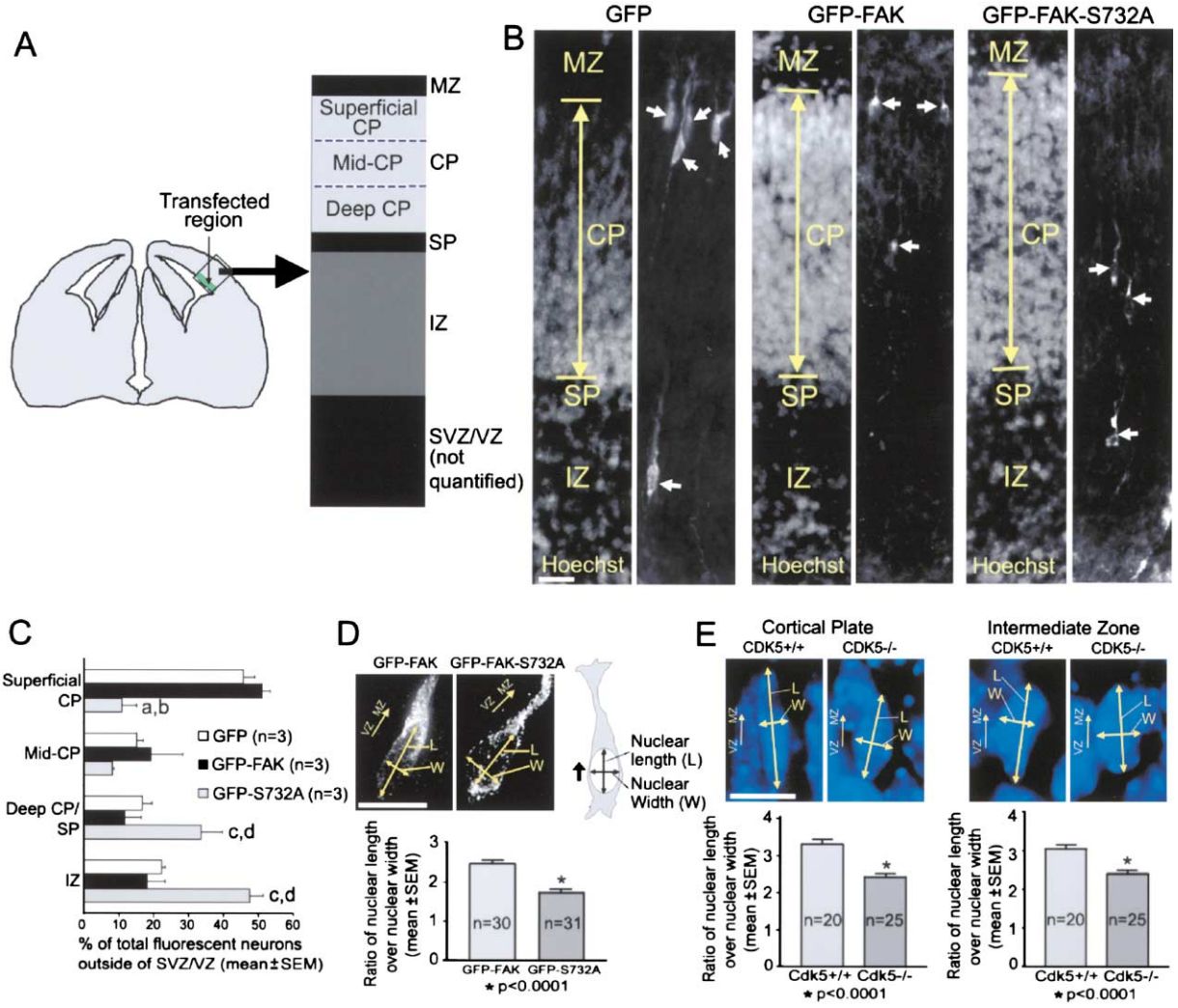

Figure 6. Expression of GFP-FAK-S732A in the Developing Cortex Results in Altered Neuronal Positioning and Abnormal Nuclear Morphology Reminiscent of Cdk5 Deficiency

(A) Region transfected by in utero electroporation is indicated. Abbreviations: MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

(B) Coronal cryosections electroporated with indicated plasmid at E14 and sacrificed at E17 were stained with a GFP antibody and Hoechst 33258. Arrows indicate transfected cells.

(C) Three different experiments each for GFP, GFP-FAK, and GFP-FAK-S732A, with 30–150 cells counted per experiment. Cells in the SVZ and VZ were not counted. Significantly ($p < 0.001$, one way ANOVA) less neurons expressing GFP-FAK-S732A reached the superficial CP relative to neurons expressing GFP (a) or GFP-FAK (b). Significantly ($p < 0.05$, one way ANOVA) more neurons expressing GFP-FAK-S732A stayed in the deep CP/SP and IZ relative to GFP (c) and GFP-FAK (d).

(D) The nuclear length (L) and nuclear width (W) is assigned relative to the leading process, which points to the MZ. The L/W ratio is significantly lower in neurons expressing GFP-FAK-S732A relative to those expressing GFP-FAK (Student's t test, $p < 0.0001$).

(E) Nuclei in coronal sections of E16 Cdk5-deficient and wild-type littermates were stained with Hoechst 33258. The L/W ratio was assessed in both the CP (left) and the IZ (right). The most elongated nuclei (top 2% of all nuclei) were used. The L/W ratio is significantly lower in $Cdk5^{-/-}$ nuclei relative to $Cdk5^{+/+}$ nuclei (student t test, $p < 0.0001$) in both brain regions. Scale bars, 20 μm in (B) and 10 μm in (D) and (E).

(Figure 7C). Immunocytochemistry of primary neocortical cultures from Cdk5-deficient mice also shows accumulation of FAK at the centrosome (Figure 7D). To confirm this result, we isolated centrosomes (Bornens and Moudjou, 1999) from brains of both Cdk5-deficient mice and wild-type or heterozygous littermates. We found

fected cell is outlined in the middle section of the bottom panel.

(E) GFP-FAK and GFP-FAK-S732A colocalize with vinculin and F-actin and do not affect the morphology of neurites and growth cones (outlined with dashed white lines).

(F) Transparent 3D reconstructed images of neocortical cultures from Cdk5 heterozygote crossing (DIV2) are shown. Arrows indicate a prominent microtubule fork in a Cdk5+/+ neuron and a disorganized microtubule structure in a $Cdk5^{-/-}$ neuron. Scale bars, 10 μm.

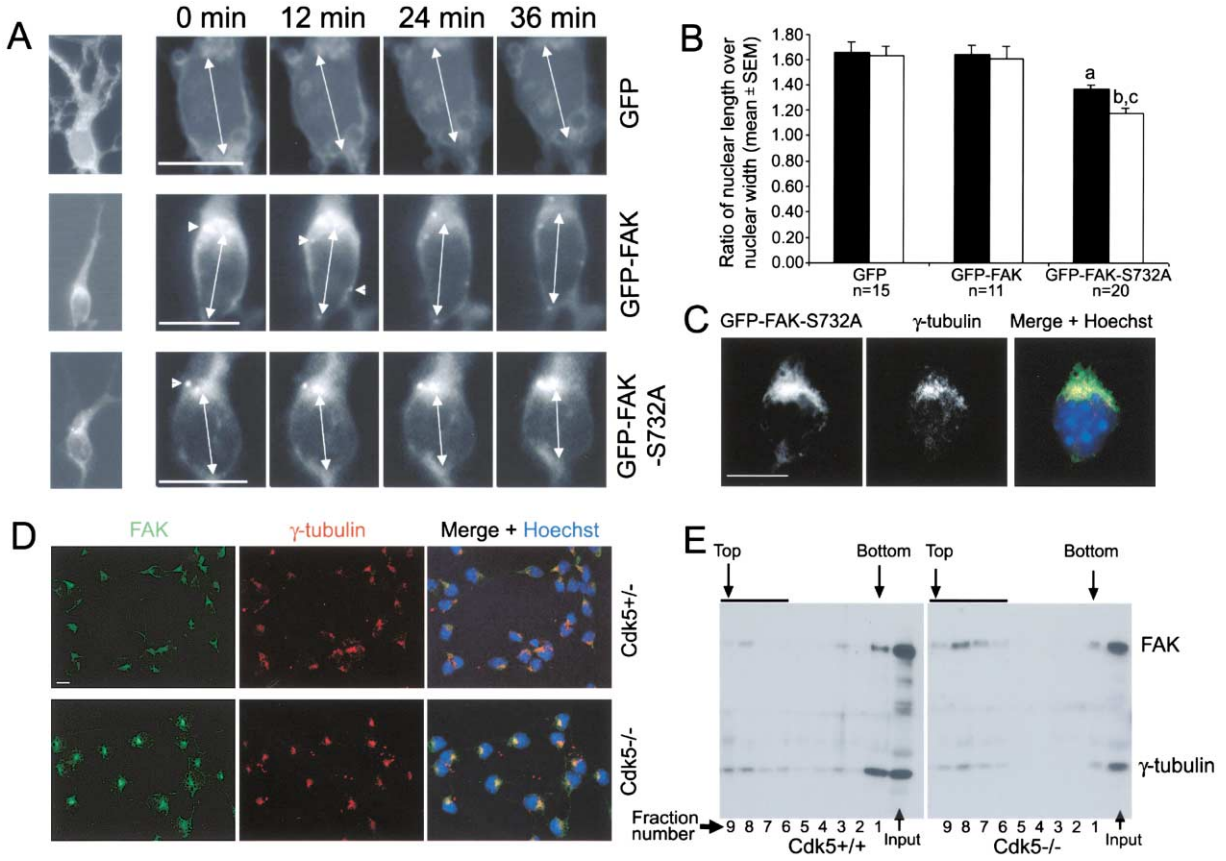

Figure 7. Overexpression of GFP-FAK-S732A Affects Nuclear Movement in Cultured Neurons

(A–C) Dissociated neocortical cultures were transfected with plasmids encoding GFP with a membrane localization domain, GFP-FAK, or GFP-FAK-S732A at DIV1 and time-lapse imaged at DIV2. Each fluorescent cell was imaged for 20–60 min. (A) Left shows the morphology of the cell, and the right shows the location and the morphology of the nucleus at different time points for cells expressing indicated plasmid. Arrows indicate nuclear length. (B) The L/W ratio was calculated as described in Figure 6D. Black columns are t1, and the white columns are the second time point, t2. At both t1 and t2, the L/W ratio of neurons expressing GFP-FAK-S732A is significantly lower (one-way ANOVA; a, $p < 0.05$ for t1; b, $p < 0.001$ for t2) relative to that expressing GFP or GFP-FAK. The L/W ratio of neurons expressing GFP-FAK-S732A is also significantly decreased at t2 relative to t1 (one-way ANOVA; c, $p < 0.05$). (C) Immunostaining of transfected neurons at DIV2 shows a neuron with morphology similar to a GFP-FAK-S732A expressing cell in the time-lapse imaging experiment accumulates GFP-FAK-S732A at a perinuclear spot enriched for γ-tubulin.

(D) Neocortical cultures from a Cdk5 heterozygote crossing were fixed at DIV2 and costained for FAK and γ-tubulin.

(E) Centrosomes were isolated and fractions containing the purified centrosomes were immunoblotted for FAK and γ-tubulin. The lines indicate fractions (around the interface of 50% and 70% sucrose) that contain purified centrosomes. The bottom fraction contains cell debris and has relatively high level of both γ-tubulin and FAK. Three pairs of $Cdk5^{-/-}$ and $Cdk5^{+/+}$, or $Cdk5^{-/-}$ and $Cdk5^{+/-}$ littermates were compared. The level of FAK was consistently higher in the $Cdk5^{-/-}$ centrosomal fractions relative to wild-type or heterozygote littermates. Scale bars, 10 μm.

significant enrichment of FAK in the centrosome fractions of Cdk5-deficient brains relative to littermates (Figure 7E). This data shows that Cdk5 phosphorylation of FAK is critical for preventing FAK accumulation in the centrosome.

Discussion

Cdk5, the reelin pathway, doublecortin, and the human type I lissencephaly gene Lis1 have been identified as major players in the regulation of neuronal positioning. To date, the signaling events downstream of these molecules that underlie neuronal migration are still unclear. In this study, we demonstrate that phosphorylation of FAK by Cdk5 is critically involved in the migration of neurons during cortical development. We show that S732 phosphorylation of FAK is important for sustaining a microtubule network, the fork-like structure that abuts the proximal portion of the nucleus in relation to the leading process of a neuron. We further show that migration of neurons is defective when the S732A mutant of FAK is expressed in the embryonic mouse brain. This FAK phosphorylation mutant also disrupts synchrony in movement of the proximal and distal poles of the nucleus, which may account for the observed migration abnormality in vivo. An accumulation of FAK at the centrosome accompanies the defects in nuclear movement. Based on these observations, we put forth the hypothesis that S732 phosphorylation of FAK by Cdk5 regulates microtubule based nuclear translocation and, in turn, neuronal migration.

FAK Is a Physiological Substrate of Cdk5

In vitro kinase assays and immunoblot analysis using a phosphospecific antibody demonstrate that Cdk5 phosphorylates FAK at S732. Phospho-S732 FAK is barely

detectable in adult brain lysates and not detectable in embryonic lysates from Cdk5-deficient mice, suggesting a developmental, Cdk5-dependent role for S732 phosphorylation. FAK is well established as a mediator of cell migration outside of the nervous system and is highly expressed in the developing brain. Our data suggests that FAK is directly downstream of Cdk5, a critical regulator of neuronal positioning. FAK is therefore a reasonable candidate to mediate some of the effects of Cdk5 during the development of the neocortex.

Cdk5 Phosphorylation of FAK at S732 Promotes the Organization of a Microtubule Fork

While most studies on FAK have demonstrated a functional role at actin-cytoskeleton-based focal adhesions, our immunostaining demonstrates that Cdk5-phosphorylated FAK decorates a microtubule structure that abuts the nucleus and is also present around the MTOC. Taken together with the microtubule preps, this data suggests that Cdk5-phosphorylated FAK is specifically enriched on microtubules.

The microtubule structure consists of two or more thick branches that abut roughly half of the top of the nucleus in cultured neurons. Since the branches usually converge at a perinuclear spot, the structure takes on a fork-like appearance. The point of convergence of the branches is the centrosome, as immunostaining shows an enrichment of γ-tubulin around this perinuclear site. Other microtubule networks around the nucleus have been described in migrating neurons, most notably a cage-like web of microtubules surrounding the nucleus of cerebellar granule cells (Rivas and Hatten, 1995). While this structure circumscribes the entire nucleus and consists of several bundles of microtubules, the fork we describe in neocortical neurons only partially encompasses the nucleus and usually consists of just a few branches.

Overexpression of a nonphosphorylatable FAK mutant or lack of Cdk5 activity suggests that phosphorylation of FAK at S732 by Cdk5 is important for promoting the organization of the fork. While the possibility that FAK regulates actin in neurons remains open, our data provides a functional role for FAK in regulating the organization of a specific microtubule structure. However, in the Cdk5-deficient brain extracts where S732 phosphorylation is abolished, the kinase activity of FAK is not consistently altered. Thus, FAK activity is probably not sufficient to sustain the integrity of the microtubule fork and phosphorylation at S732 is required. Interestingly, accumulation of FAK at the centrosome is observed in the absence of Cdk5 or with overexpression of GFP-FAK-S732A. While this accumulation may indicate that phosphorylation regulates FAK turnover, another possibility is that S732 phosphorylation is important for shuttling FAK away from the centrosome. This could be accomplished by S732 phosphorylation promoting the interaction of FAK with a protein that directs it away from the centrosome. On the other hand, S732 phosphorylation may reduce the binding of FAK to some protein that sequesters it at the centrosome. Since organization of microtubules is a prominent function of the centrosome, accumulation of FAK likely impacts on the structure of centrosome-associated microtubule networks such as the fork.

A Properly Organized Fork Is Important for Nuclear Translocation

Nucleokinesis is a well-established behavior of migrating neurons that is thought to be critically dependent on microtubules. Microtubule-dependent nuclear positioning involves a tight association between the MTOC and the nucleus (Reinsch and Gonczy, 1998). In eukaryotic cells, the centrosome is the major MTOC. We hypothesize that the microtubule fork described here is essential for achieving the tight association between the centrosome and the nucleus required for proper nucleokinesis. Three lines of evidence support this hypothesis. First, immunostaining shows that the fork provides a direct link, as its branches abut the nucleus and converge at the centrosome. Secondly, when the S732A mutant is overexpressed and the fork is disorganized, time-lapse imaging demonstrates impaired nuclear movement. Third, Lis1, Nudel, and Dynein, whose homologs control nuclear distribution in *Aspergillus nidulans* (Lambert de Rouvroit and Goffinet, 2001; Morris et al., 1998), all localize to the fork.

Neurons that overexpress FAK-S732A show a difference in movement between the poles of the nucleus proximal and distal to a thick process. While the distal portion of the nucleus begins to translocate, the proximal pole fails to move in tandem. This results in a more rounded nuclear morphology, suggesting that the phospho-FAK organized microtubule fork is important specifically for movement of the nuclear pole proximal to the leading process, but not the distal pole. This idea is supported by the immunostaining showing that the fork does not abut the entire nucleus, but roughly only the half proximal to a thick process. It is possible that a properly organized fork lying directly on the nucleus and tethered to the centrosome grasps and pulls the proximal pole of the nucleus in the leading process. This suggests that distinct mechanisms regulate translocation of different nuclear poles.

FAK Phosphorylation by Cdk5 Regulates Neuronal Migration

In utero electroporation of GFP-FAK-S732A into the developing neocortex of embryonic mouse brains demonstrates a phenotype where neurons do not migrate fully to their appropriate position in the cortical plate. Three pieces of evidence suggest that effects of FAK-S732A are mediated during migration. First, in embryonic mouse brain sections, phospho-FAK mostly colocalizes with Hu, a marker for postmitotic neurons. Second, in vitro time-lapse imaging demonstrates a major impairment in nuclear movement, a critical process during neuronal migration. Third, neurons electroporated in vivo display an abnormal, rounded nuclear morphology consistent with the nuclear movement defects seen in vitro. The trademark of mouse brains deficient in Cdk5 is an impaired cytoarchitecture of the neocortex, a phenotype that is recapitulated with overexpression of FAK-S732A. This implies that phosphorylation of FAK is important for mediating the effects of Cdk5 during neocortical development.

A Biological Perspective of the Role of Cdk5 and FAK in Neuronal Migration

Here, we present a mechanism, phosphorylation of FAK, which suggests a role for Cdk5 in the regulation of

nucleokinesis. The full effects of Cdk5 during neuronal migration, however, may be mediated by several mechanisms. For instance, Cdk5 may regulate the dynamics of the actin cytoskeleton through small GTPases (Nikolic et al., 1998) and neuronal adhesion through the N-cadherin/β-catenin complex (Kesavapany et al., 2001; Kwon et al., 2000).

Hints as to other mechanisms regulating nucleokinesis during mammalian neuronal migration come from the slime mold *Aspergillus nidulans*, where several mutations (abbreviated "Nud") that also result in abnormal nuclear distribution have been described (Lambert de Rouvroit and Goffinet, 2001; Morris et al., 1998). The homolog of NudF is Lis1, one of the genetic origins of type I lissencephalies (Reiner et al., 1993; Reiner and Sapir, 1998; Xiang et al., 1995). Cytoplasmic components of the retrograde motor dynein also have homologs (NudA, NudC, and NudG) that, when mutated, result in abnormal nuclear distribution in *Aspergillus* (Beckwith et al., 1998; Xiang et al., 1994; Xiang et al., 1999). A link between Lis1 and Dynein is found with either mNudE or the NudE-like protein Nudel, which is also a Cdk5 substrate (Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000). Nudel is highly enriched around the centrosome and may play a conserved function in regulating nuclear translocation. As FAK, Nudel, Lis1, and Dynein all localize to the microtubule fork, FAK and Nudel together may provide Cdk5 with different pathways by which to dynamically regulate nucleokinesis and ultimately, neuronal migration.

In sum, we have uncovered a mechanism by which Cdk5 regulates neuronal migration. Phosphorylation of FAK at S732 is important for organization of a microtubule fork that may function to pull the proximal region of the nucleus into the leading process. If FAK cannot be phosphorylated by Cdk5, the fork becomes disorganized, the proximal pole of the nucleus fails to translocate properly, and impairments of neuronal migration result. While phosphorylation of FAK is an important step in understanding the mechanisms that underlie Cdk5 control of neuronal migration, other mechanisms that further mediate the effects of Cdk5 remain to be discovered.

Experimental Procedures

DNA Constructs

pRc/CMV-FAK-HA (Calalb et al., 1995) is from S. Hanks. Constructs expressing GST-FAK-N, GST-FAK-KD, and GST-FAK-3 were created by inserting the corresponding fragments derived via PCR into the vector pGEX-3X. The construct expressing GST-FAK-C is from B. Eliceiri. FAK-HA derived from pRc/CMV-FAK-HA was inserted into the vector pEGFP-C1 to produce a construct encoding GFP-FAK. GST-FAK-3-S722A, GST-FAK-3-S732A, and GFP-FAK-S732A were made by PCR mutagenesis that replaced S722 or S732 with an alanine. All constructs were verified by sequencing. The plasmids for Cdk5, p35, and p25 have been described (Patrick et al., 1999).

Antibodies

To generate the phospho-S732 antibody, rabbit antiserum was raised against the phosphopeptide SEGFYPpSPQHMVC (residues 726–737 of mouse FAK with the addition of a cysteine at the C terminus). The antiserum was preabsorbed through the cognate nonphosphopeptide column and then affinity purified with the phospho-S732 peptide column using a SulfoLink kit (Pierce, Rockford, IL). The following antibodies were also used: FAK 2A7 (Upstate Biotechnology, Lake Placid, NY), FAK C-20, and Dynein IC (Santa Cruz Biotechnology, Santa Cruz, CA); pS722-FAK (Biosource, Camarillo, CA), β III tubulin (Tuj1) (Covance, Richmond, CA), α-tubulin, γ-tubulin GTU-88, and γ-tubulin polyclonal (Sigma, St. Louis, MO); and Hu and GFP (Molecular Probes, Eugene, OR). Antibodies against Nudel, pS231-Nudel, and Lis1 have been described (Niethammer et al., 2000; Patzke et al., 2003; Smith et al., 2000).

Preparation of Brain Lysates, Western Blot, and Immunoprecipitation

Mouse embryonic brains were homogenized in radioimmunoprecipitation buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibtors). For Western blot analysis, lysates were either used fresh, or stored at −80°C until use. For immunoprecipitation, lysates were incubated with antibodies at 4°C for 1 hr, and the immunocomplex was isolated by protein G-Sepharose (Amersham Pharmacia, Uppsala, Sweden).

In Vitro Kinase Assay

5 μg of FAK fragments fused with GST were incubated with p35/Cdk5 (2 μl) produced in bacculovirus or GST-p25 (1 μg)/GST-Cdk5 (1 μg) purified from bacteria in kinase buffer (30 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) supplemented with 100 μM cold ATP and 5 μCi [^{32}P]γATP in a final volume of 50 μl for 30 min at room temperature. The reaction was terminated by the addition of 50 μl of 2× sample buffer. Samples were separated by SDS-PAGE and [^{32}P]γATP incorporation was assessed by autoradiography.

Neocortical Culture and Transfection

Primary culture and transfection of neurons were performed as described (Niethammer et al., 2000) except that neocortical cells from E15–17 mice were used for culture, cells were maintained in neurobasal medium (GIBCO, Grand Island, NY) supplemented with B27 and 1 mM L-glutamine, and calcium phosphate transfection was performed 20 hr after plating.

In Utero Electroporation

In utero electroporation was performed as described (Tabata and Nakajima, 2001) on E14 mice. Embryos were sacrificed 72 hr after electroporation.

Immunohistochemistry, Immunocytochemistry, and CIP Treatment

Dissected embryonic brains were fixed in PBS containing 4% paraformaldehyde for 3–4 hr at 4°C, soaked in PBS containing 20% sucrose overnight at 4°C, and embedded in OCT compound (Sakura Tissue Tek, Torrance, CA). 12 μm cryosections were used for anti-pS732 immunostaining. 40 μm cryosections were used for anti-GFP immunohistochemistry on electroporated embryos. Cells cultured on glass coverslips were fixed in PBS containing 4% paraformaldehyde for 5 min at room temperature and processed for immunostaining. For CIP treatment, following permeabilization with PBS containing 3% BSA and 0.2% Triton X-100, samples were washed in phosphatase buffer (50 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$) and then incubated at 37°C in phosphatase buffer supplemented with 200 unit/ml of CIP or with H_2O (as a control) for 2 hr (for brain sections) or 40 min (for cultured cells on coverslips) prior to immunostaining.

Imaging

Confocal microscopy was performed using a Zeiss LSM 510 system (Zeiss, Thornwood, NY) at Harvard Center for Neurodegeneration and Repair. The 3D reconstructions of confocal images used LSM 5 Image Examiner software (Zeiss, Thornwood, NY) or Volocity (Improvision, Lexington, MA). Z series images of transfected cells from in utero electroporations were obtained on a Nikon inverted microscope linked to a DeltaVision deconvolution system (Applied Precision, Seattle, WA) and 3D reconstruction of Z series images was performed using softWoRx (Applied Precision, Seattle, WA). For time-lapse imaging, neurons were incubated in imaging media (sodium bicarbonate-free DMEM/F12 (GIBCO, Grand Island, NY) supplemented with N2, 1 mM Glutamine, 25 mM HEPES, 1 mM pyruvic acid, 0.1% ovalbumin, 0.6% glucose) in a dish whose temperature

was maintained at 37°C by a circulating waterbath (VWR Scientific, West Chester, PA) and were viewed on a Nikon inverted microscope linked to a SenSys air-cooled, charge-coupled-device camera. The time-lapse programming and processing of the imaging was performed using Openlab system (Improvision, Lexington, MA).

Centrosome Preparation

Centrosomes were isolated from mouse embryonic brains by a procedure modified from that described (Bornens and Moudjou, 1999). Embryonic brains were lysed (1 mM Hepes [pH7.2], 0.1% NP-40, 0.5 mM $MgCl_2$, 0.2 mM Tris-HCl [pH 7.4], 1.5 mM NaCl, 0.8% sucrose, 0.1% β-mercaptoethanol with protease and phosphatase inhibitors) and centrifuged at 2500 × g for 10 min. The supernatant was loaded on top of a step sucrose gradient in a 4 ml ultracentrifuge tube (from the bottom: 1.5 ml of 70% sucrose, 0.9 ml of 50% sucrose, 0.9 ml of 40% sucrose). The sucrose solution was made in buffer containing 10 mM Pipes [pH 7.2], 0.1% Triton X-100, and 0.1% β-mercaptoethanol. Samples were centrifuged at 40,000 × g for 2 hr at 4°C and fractions from the bottom to the regions around the interface of the 50% and 70% sucrose gradient were collected and analyzed.

Microtubule Preparation

The MAP-rich tubulin preparation from bovine brain was purchased from Cytoskeleton, Inc. (Denver, CO). For microtubule preparation, COS7 cells overexpressing p35 and Cdk5 were lysed (50 mM Pipes-NaOH, 50 mM Hepes [pH 7.0], 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors) and centrifuged at 24,000 × g for 30 min at 4°C. The supernatant was collected and centrifuged at 150,000 × g for 60 min at 4°C. The supernatant was then incubated at 37°C for 12 min in the presence of 20 μM of taxol. Following incubation, the mixture was centrifuged at 40,000 × g for 30 min at 35°C on top of a 0.5 volume 7.5% sucrose solution. The pellet was resuspended in extraction buffer as microtubule pellet. The supernatant was saved as microtubule supernatant.

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