Doublecortin Microtubule Affinity Is Regulated by a Balance of Kinase and Phosphatase Activity at the Leading Edge of Migrating Neurons

Bruce T. Schaar,[1] Kazuhisa Kinoshita,[2]
and Susan K. McConnell[1,*]
[1]Department of Biological Sciences
Stanford University
Stanford, California 94305
[2]Max Plank Institute of Molecular Cell Biology
    and Genetics
Dresden 01307
Germany

Summary

Doublecortin (Dcx) is a microtubule-associated protein that is mutated in X-linked lissencephaly (X-LIS), a neuronal migration disorder associated with epilepsy and mental retardation. Although Dcx can bind ubiquitously to microtubules in nonneuronal cells, Dcx is highly enriched in the leading processes of migrating neurons and the growth cone region of differentiating neurons. We present evidence that Dcx/microtubule interactions are negatively controlled by Protein Kinase A (PKA) and the MARK/PAR-1 family of protein kinases. In addition to a consensus MARK site, we identified a serine within a novel sequence that is crucial for the PKA- and MARK-dependent regulation of Dcx's microtubule binding activity in vitro. This serine is mutated in two families affected by X-LIS. Immunostaining neurons with an antibody that recognizes phosphorylated substrates of MARK supports the conclusion that Dcx localization and function are regulated at the leading edge of migrating cells by a balance of kinase and phosphatase activity.

Introduction

The development of the mammalian cortex is a highly orchestrated process involving coordinated cell division, migration, and the expression of layer-specific neuronal phenotypes (McConnell, 1995). Following the division of progenitor cells within the ventricular epithelium, a subset of daughter cells differentiate into postmitotic neurons, exit the proliferative zone, and migrate outward toward the pial surface, where they will occupy the most superficial layer of the developing cortical plate. Subsequent cohorts of young neurons migrate beyond the previously settled layers in an inside-first, outside-last procession until six histological layers are formed and the ventricular zone is depleted of neuronal progenitors (Angevine and Sidman, 1961).

Mounting evidence suggests that migrating neurons navigate the journey from their origin to their final destination by utilizing attractive and repulsive cues, cell surface receptors, and cytoskeletal elements that are similar to those used during axon guidance, another highly stereotyped cell motility event (Ward et al., 2003; Wu et al., 1999). Although steady progress has been made

in identifying guidance cues and their receptors, the question of how these signals converge onto ubiquitous cytoskeletal components, such as actin, microtubules, and their associated proteins, to bring about directed migration is still quite open. The role of microtubules in axon growth and guidance has been characterized primarily through pharmacological studies, which have revealed that microtubule dynamics are required for the growth cone to turn toward a chemoattractant or away from a repulsive cue (Challacombe et al., 1997; Lin et al., 1994; Tanaka et al., 1995; Tanaka and Kirschner, 1995; Williamson et al., 1996). One interpretation of these observations is that microtubules simply add structural integrity to the growth cone. However, recent studies have demonstrated that perturbing microtubule dynamics can affect both actin dynamics and membrane fusion, indicating a broad role of microtubules in regulating cell motility (Rochlin et al., 1999; Rodriguez et al., 2003; Zakharenko and Popov, 1998).

The role of microtubules in neuronal migration during cortical development has been highlighted by the recent identification of two genes that are mutated in human lissencephaly. The primary developmental dysfunction in type I lissencephaly ("smooth brain") is a defect in neuronal migration, which results in the absence of sulci and gyri in the mature brain, giving it a smooth appearance. Symptoms include epilepsy and mental retardation (Feng and Walsh, 2001; Gleeson, 2000). One form of lissencephaly results from an autosomal haploinsufficiency in *LIS1*, which encodes a conserved regulator of the microtubule motor dynein (Faulkner et al., 2000; Reiner et al., 1993; Smith et al., 2000). A second form of lissencephaly is X linked and yields a phenotype in affected males that is grossly similar to a *LIS1* mutation. However, female patients carrying the mutation show an unusual neuropathology called subventricular band heterotopia or doublecortex syndrome, in which a band of gray matter underlies a normal-looking region of the cortex. Correspondingly, the affected gene has been named *Doublecortin* (*DCX*) (Gleeson et al., 1998; des Portes et al., 1998).

Several lines of evidence have shown that Dcx protein is a novel microtubule-associated protein (MAP) that interacts with and regulates the microtubule cytoskeleton (Francis et al., 1999; Gleeson et al., 1999). Dcx binds to and stabilizes microtubules in vitro and will bundle microtubules in transfected cells (Yoshiura et al., 2000). The protein contains two repeats called Dcx domains that directly mediate microtubule binding and define a novel family of MAPs. Patient missense mutations neatly delineate the tandem Dcx repeats, providing genetic evidence that the function of Dcx in neuronal migration depends on its ability to bind and stabilize microtubules (Sapir et al., 2000; Taylor et al., 2000). A recent structural and functional analysis of the two Dcx domains has provided evidence suggestive of somewhat distinct functional properties: the N-terminal domain binds to microtubule polymers, whereas the C-terminal domain binds both tubulin dimers and microtubules (Kim et al., 2003). Collectively these data raise the possibility that

*Correspondence: suemcc@stanford.edu

Dcx may utilize its C-terminal Dcx domain to catalyze microtubule growth by stimulating the addition of tubulin dimers to the growing tip and use both domains together to bundle or stabilize the microtubule polymer, protecting it from catastrophic collapse.

Consistent with this notion, we have found that Dcx is localized in migrating neurons to a region in which microtubule dynamics likely play a key role in motility and guidance. Similar to the growth cone of an axon or neurite, migrating neurons extend a leading process along their direction of movement. The two structures play analogous roles in motility, with a major difference being that during migration the cell soma is translocated in a saltatory manner following extension of the leading process, whereas during axon growth the cell body remains stationary (Edmondson and Hatten, 1987; O'Rourke et al., 1992; Wichterle et al., 1997). Strikingly, Dcx does not bind microtubules throughout the neuron but is rather associated stably and selectively with microtubules found in the growth cones of neurites and the leading processes of migrating neurons. Previous studies have shown that microtubules are dynamic in the distal regions of growth cones and become bundled at the boundary of the neurite shaft (Brown et al., 1993; Lin et al., 1994; Rivas and Hatten, 1995; Tanaka et al., 1995). Dcx localizes to both of these areas but is not associated with microtubules in the regions of the neurite and leading process located more proximally toward the nucleus. Dcx is thus perfectly poised to regulate microtubule growth and stability in the leading processes of migrating neurons.

Both the subcellular localization of Dcx and its proposed role in migration necessitate that the microtubule binding activity of Dcx be subject to regulation. Although microtubules in growth cones and leading processes are continuous with those in the axon and migrating neuron, respectively, Dcx binds to microtubules preferentially in distal regions. Thus, microtubule binding is either stimulated within the leading process or prevented in more proximal regions. Furthermore, the dynamic behavior of microtubules in growth cones, and what we assume to be a similar set of behaviors in leading processes, suggest that the microtubule binding activity of Dcx is subject to dynamic regulation that enables motile processes to alter their trajectory in response to extrinsic guidance cues. We have focused on phosphorylation as a mechanism for regulating Dcx localization and function. Here we show that the subcellular localization of Dcx is maintained by a balance of phosphatase and kinase activities at the leading edge of the migrating neuron. We find that the binding of Dcx to microtubules in vitro is negatively regulated by PKA and MARK kinase activities. Dcx localization is complementary to that of phosphorylated substrates of the MARK, a protein whose orthologs in *C. elegans* (PAR-1) and *Drosophila* (dPAR-1) also orchestrate cytoskeletal organization and cell polarity (Drewes et al., 1997; Drewes and Mandelkow, 1998; Guo and Kemphues, 1995; Shulman et al., 2000). Finally, we identified a serine residue that is required for the regulation of Dcx microtubule binding by MARK and PKA in vitro, and we find that this serine is a target of missense mutations in patients affected by X-LIS. These data provide strong support for the hypothesis that the phosphoregulation

of Dcx is crucial for its function in cell migration. While preparing this paper for publication, we learned of complementary data from Gleeson and colleagues (see the accompanying paper by Tanaka et al.), which demonstrate a role for Cdk5 in regulating the binding affinity of Dcx for microtubules in the perinuclear region of the migrating cell. Collectively these studies suggest that Dcx can regulate microtubule behavior in multiple subcellular locations and that several kinases govern Dcx activity.

Results

Dcx Is Localized to Distal Microtubules in Neurites and the Leading Processes of Migrating Neurons

When embryonic cortical neurons are dissociated and grown on laminin- or polylysine-coated substrates, the cells differentiate rapidly and extend neurites. Dcx is highly concentrated at the tips of these growing neurites (Figure 1A). When cultured neurons are extracted under conditions that remove soluble proteins and stabilize existing microtubules, Dcx colocalizes with distal microtubules in the growth cone but is not associated with microtubules in the neurite more proximal to the cell body (Figure 1B). Western blot analysis of extracted proteins and insoluble material under these conditions indicates that Dcx is present in approximately equal amounts in both fractions (data not shown), consistent with the observation by others that a significant fraction of Dcx cofractionates with membranes (Kizhatil et al., 2002). We find that under different fixation conditions, more Dcx is found associated with the cell soma; however, we have focused on the subpopulation of Dcx localized to the leading edge of neurites and migrating neurons.

In order to visualize the subcellular localization of Dcx in migrating neurons, explants from the postnatal rat anterior subventricular zone (SVZa) were cultured overnight in Matrigel. SVZa neurons express Dcx throughout the life of the rodent, as the cells migrate though the rostral migratory stream (Wichterle et al., 1997). When cells are fixed as above to extract soluble proteins and stabilize the cytoskeleton, Dcx immunostaining localizes to the microtubules of the leading edge of the migratory process, as well as to the area just distal to the leading edge (Figure 1C). Relatively little Dcx immunoreactivity was detected in the cell body under these fixation conditions. Thus, Dcx interacts selectively with microtubules within the leading process, positioning Dcx to play a key role in regulating the cytoskeleton during motility and guidance.

Phosphatase Activity Maintains Dcx Localization at Neurite Tips

Because the localization patterns of Dcx in the leading processes of migrating neurons and the growth cones of extending neurites are quite similar, cultured embryonic rat dissociated cortical neurons were used to explore the mechanisms that control Dcx's subcellular localization. Dcx binds microtubules avidly in vitro and when transfected into fibroblasts, suggesting that its microtubule binding activity must be regulated locally
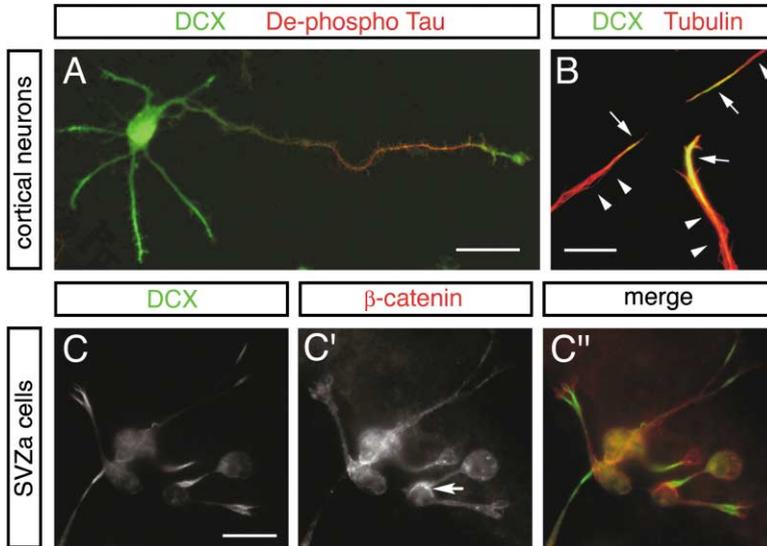
Figure 1. Doublecortin Localizes to the Tips of Neurites and Leading Processes of Migrating Neurons

(A) Dcx (green) localizes to distal regions of axons as well as the dendrites and cell bodies of cultured cortical neurons fixed in 4% paraformaldehyde. The axon is stained with antibodies that recognize unphosphorylated Tau (red). Scale bar equals 25 μm.

(B) Dcx (green) colocalizes with microtubules (red) at the tips of cortical neurites (arrows) that were pre-extracted under microtubule stabilizing conditions and fixed in methanol. Dcx (arrows) is not associated with microtubules in the neurite shaft more proximal to the cell body (arrowheads). Scale bar equals 5 μm.

(C) Dcx is localized within the leading processes of SVZa cells migrating in a Matrigel explant.

(C′) Antibodies against β-catenin demarcate the cell surface and highlight points of cell-cell contact (arrow).

(C″) Dcx (green) is enriched within leading processes, but staining does not extend to the leading edge of the process, which is labeled with β-catenin (red).

within neurons. Because phosphorylation modulates the microtubule affinity of several MAPs (Cassimeris, 1999), we hypothesized that the phosphorylation state of Dcx regulates its localization to growth cone microtubules in intact neurons.

Dcx localization was studied following treatment with several cell-permeant phosphatase and kinase inhibitors. The most striking effects were seen when neurons were treated with 10 nm Okadaic acid, which inhibits Protein Phosphatase 2A (PP2A) at this low concentration. To minimize the pleiotropic effects of Okadaic acid on neurons in culture, cells were examined at short times after exposure, prior to the atrophy of neurites that occurs under prolonged exposure to this agent. After 5–10 min of exposure to 10 nm Okadaic acid, Dcx is lost from the tips of most neurites, and immunoreactivity increases in the cell soma (Figures 2A and 2B). Tests of cell viability suggest that the delocalization of Dcx is not due to cell toxicity. Western blots confirm that Dcx

mobility on SDS-PAGE is retarded compared to control (DMSO) and nocodazole (5 μg/ml, which causes rapid Dcx dephosphorylation) treatments (Figure 2C). Thus, inhibition of PP2A in neurons causes a rapid shift in Dcx localization, suggesting that Dcx function may be spatially regulated by a balance of kinase and phosphatase activity.

Dcx Microtubule Binding Is Inhibited by MARK and PKA In Vitro

Inspection of the Dcx amino acid sequence revealed a consensus phosphorylation site (KIGS) for MARK (MAP/Microtubule Affinity Regulatory Kinase), a kinase that negatively regulates the microtubule affinity of several MAPs that contain this sequence in their microtubule binding domains (Drewes and Mandelkow, 1998, 1999). The MARK kinase family includes the *C. elegans* kinase PAR-1, which regulates cellular asymmetry during em-
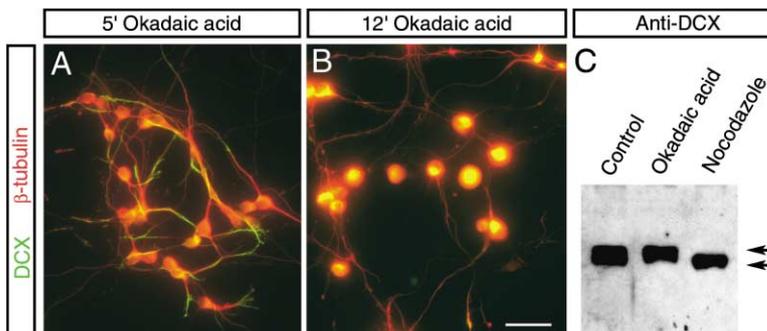
Figure 2. Displacement of Doublecortin from Neurite Tips by Limited Okadaic Acid Treatment

Brief treatment of cortical neuron cultures with 10 nM Okadaic acid causes the loss of Dcx from neurite tips and relocalization to the cell soma. Cells were fixed with paraformaldehyde following Okadaic acid treatment.

(A) Neurons treated for 7 min with Okadaic acid show Dcx staining (green) at neurite tips. Antibodies against tubulin (red) reveal neurites.

(B) After a 12 min treatment with 10 nM Okadaic acid, neurites are still intact (red), but Dcx has disappeared from neurite tips. The bulk of Dcx immunoreactivity appears in neuronal cell bodies. Scale bar equals 20 μm.

(C) Western blots of cortical neuron extracts subjected to SDS-PAGE and probed with antibodies against Dcx reveal that Dcx migrates as a pair of bands under control conditions (0.4% DMSO). Okadaic acid treatment causes Dcx to migrate more slowly (top arrow), presumably due to the enrichment of phosphorylated forms of the protein. Nocodazole treatment (5 μg/ml, which causes rapid Dcx dephosphorylation) results in enrichment of the more rapidly migrating species (bottom arrow).
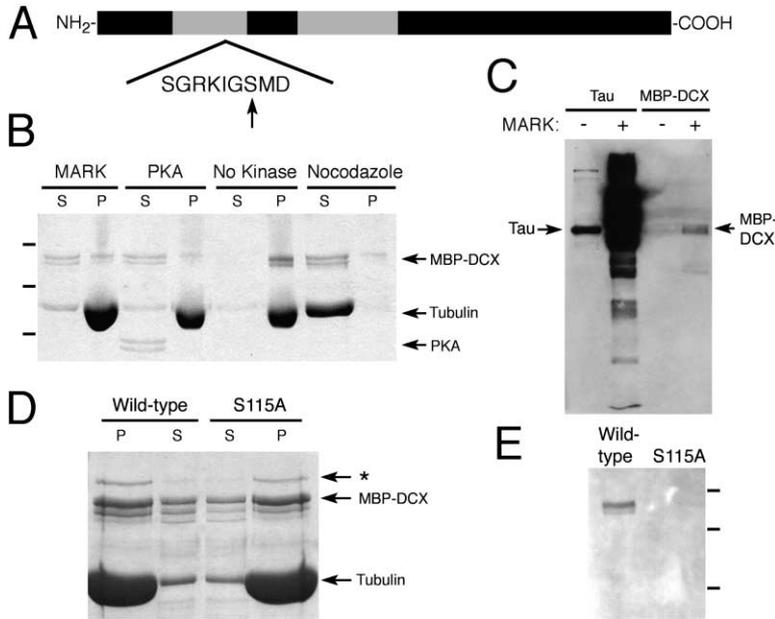
Figure 3. Direct Inhibition of Doublecortin-Microtubule Interactions by MARK and PKA

(A) Diagram of Dcx showing the two microtubule binding repeats (gray boxes) and the putative MARK site KIGS.

(B) Phosphorylation by either MARK or PKA can prevent microtubule binding of a Dcx fusion protein in vitro. Gels containing the supernatant (S) and pelleted fractions (P) from microtubule cosedimentation assays were stained with Coomassie blue. The MBP-Dcx fusion protein appears as a doublet due to proteolysis, but this does not affect microtubule binding in the absence of kinase. Under the latter conditions, nearly all MBP-Dcx is found in the pellet. In the presence of MARK or PKA, ~50% of the fusion protein is prevented from binding microtubules and is found in the supernatant. Nocodazole prevents polymerization of microtubules and Dcx is present only in the supernatant.

(C) Western analysis using 12E8 antibody on purified Tau and MBP-Dcx proteins in the presence or absence of MARK. Phosphorylation of MBP-Dcx or Tau by MARK generates the phosphoepitope recognized by 12E8. Similar results were obtained after PKA treatment (not shown).

(D) Mutation of the KIGS site to KIGA (S115A) does not affect Dcx's microtubule affinity after kinase treatment. Both fusion proteins were treated with MARK and subjected to microtubule cosedimentation as in (B). The asterisk indicates a slower migrating species of the fusion protein found after kinase treatment.

(E) The S115A mutation abolishes 12E8 phospho-dependent immunoreactivity. 10% of the supernatants in (D) were subjected to Western analysis with 12E8 antibody. Protein standards in (B) and (E) are 107, 67, and 44 kDa.

bryogenesis (Guo and Kemphues, 1995). MARK2 has also been implicated in neuronal polarity in a neuroblastoma cell line (Biernat et al., 2002). The KIGS site in Dcx (amino acids 112–115, found within the first repeat required for microtubule binding; Figure 3A) provides an attractive candidate for regulating Dcx function for several reasons. First, multiple copies of a KIGS motif are present in other MAPs, and mutation of these sites to KIGA abolishes regulation of microtubule binding by MARK-dependent phosphorylation (Drewes and Mandelkow, 1999; Illenberger et al., 1996). Second, MARK-1 and -2 isoforms are expressed in the same cells and developmental time period as Dcx in the cortex (Bessone et al., 1999; Drewes et al., 1997). Finally, the KIGS sequence can also serve as a substrate for cAMP-regulated Protein Kinase A (PKA), albeit at lower efficiency (Drewes et al., 1997). PKA is also a good candidate for regulating Dcx, since cAMP levels affect the interpretation by growth cones of axon guidance ligands such as Netrin as either attractive or repulsive cues (Ming et al., 1997; Song et al., 1997).

To ascertain whether MARK and/or PKA can regulate the binding affinity of Dcx for microtubules, a purified bacterial fusion protein of Maltose Binding Protein (MBP) and Dcx was used as a substrate for each kinase, and the phosphorylated fusion protein was tested for its ability to cosediment with taxol-stabilized microtubules. Normally, 100% of MBP-Dcx fusion protein cosediments with taxol-stabilized microtubules (Figure 3B, no kinase). However, if the MBP-Dcx is first incubated with ATP and either purified MARK or PKA, roughly 50% of the MBP-Dcx remains in the supernatant and does not

bind to microtubules (Figure 3B). Other candidate kinases such as CaMKII and Protein Kinase G produced no effect on the affinity of MBP-Dcx for microtubules in this assay (data not shown).

To assess the role of the MARK consensus sequence in regulating microtubule affinity, we first asked if phosphorylation of MBP-Dcx generates a phosphoepitope recognized by the monoclonal antibody 12E8. Originally generated against a phosphoepitope of the KIGS sequence in Tau (Seubert et al., 1995), the antibody also recognizes phosphorylated KIGS sites in the context of other MAPs (Drewes and Mandelkow, 1999). Phosphorylation of MBP-Dcx by MARK or PKA generates the phosphoepitope recognized by 12E8 (Figure 3C). Tau was used as a positive control in this assay; it is much more reactive because it contains four KIGS sequences. We then mutated the KIGS site in Dcx to KIGA (S115A mutation) and asked whether this fusion protein can still be regulated by MARK and PKA. Strikingly, this mutation does not visibly disrupt the ability of MARK or PKA to trigger a decrease in microtubule binding. Using the cosedimentation assay, there was no difference in the amount of S115A mutant fusion protein compared to wild-type Dcx in the supernatant fraction following phosphorylation in vitro (Figure 3D). To confirm that the consensus MARK site was truly ablated in the S115A mutant, one-tenth of the supernatant fractions from both the mutant S115A and wild-type Dcx cosedimentation assays were subjected to Western analysis using the 12E8 antibody. As expected, Dcx containing the S115A mutation fails to show immunoreactivity for the phosphoepitope following kinase treatment (Figure 3E).

A Mass spectroscopy of unbound
MBP-DCX tryptic digests:

(R)TRTLQALSNEKK(R):
Non-Phosphorylated: 1387.74
PKA-Kinased: 1468.89
MARK-Kinased: 1468.84

(R)TRTLQALSNEK(K):
Non-Phosphorylated: 1260.69
PKA-Kinased: 1340.70
MARK-Kinased: 1336.70

B

KIGS

NH₂- ▬▬▬▬▬▬▬▬▬ -COOH

TRTLQALSNEKKA

C

Figure 4. Identification of Serine 47 as a Crucial Residue for Negative Regulation

(A) Mass spectroscopy was performed on tryptic peptides from fractions of S115A MBP-Dcx that was not bound to microtubules after MARK and PKA treatment, and on peptides from MBP-Dcx in the absence of kinase. Two tryptic fragments (differing by a single lysine) from kinase-treated MBP-Dcx showed spectral changes consistent with phosphorylation.

(B) Location of S47 in the first microtubule binding domain of Dcx. The location of the KIGS site is also indicated.

(C) Mutation of S47 to alanine inhibits kinase regulation of Dcx but does not affect microtubule binding. Microtubule binding by wild-type MBP-Dcx (lanes 3–4) is inhibited by pretreatment with MARK. In the absence of kinase, the S47A/S115A mutant protein binds avidly to microtubules (lanes 5–6). However, in the presence of MARK (lanes 1–2), S47A/S115A protein remains in the pelleted fraction. Similarly, MBP-Dcx containing the single substitution S47A remains bound to microtubules in the presence (lanes 9–10) or absence (lanes 7–8) of PKA. Thus, the S47A mutation is sufficient to prevent regulation by PKA in vitro.

Identification of S47 as a Critical Target of MARK and PKA

We then sought to identify the other site(s) critical for MARK and PKA to regulate the binding affinity of Dcx for microtubules. Dcx exists in vivo as a large number of phospho-isoforms, indicating that numerous potential combinations of phosphorylation sites may be utilized. To identify critical residues that must be phosphorylated to prevent Dcx from binding microtubules, MARK- and PKA-phosphorylated S115A mutant fusion proteins that remained in the supernatant after a microtubule cosedimentation assay were subjected separately to tryptic digestion and mass spectroscopy analysis, as was unphosphorylated MBP-Dcx. We then compared the spectra of these three samples, under the rationale that if a phosphorylated site is critical, it should be present in both of the mutant fusion proteins following kinase treatment, regardless of whether the kinase was MARK or PKA, and there should not be a detectable peak corresponding to the unphosphorylated form of this peptide in either kinase reaction. Two peptides (amino acids 42–50 and 42–51) conformed to these criteria (Figure 4A). The peptides differed in only a single lysine residue, due to alternative cleavage by trypsin, and each contained a single serine residue (S47).

A search of known missense mutations in Dcx revealed that S47 is the site of a missense mutation in two independent cases of X-LIS (Gleeson et al., 1998; Kim et al., 2003; des Portes et al., 1998; F. Francis, personal communication), indicating the functional importance of this residue in vivo. S47 marks the beginning of the first microtubule binding repeat (Figure 4B). To ascertain whether S47 is required for regulation of Dcx's microtubule affinity by MARK and PKA, we mutated S47 to alanine in the S115A mutant, subjected the resulting MBP fusion protein to in vitro kinase reactions with MARK and PKA, and then performed microtubule cosedimentation assays. Although the S47A/S115A double mutant protein binds to microtubules quantitatively in this assay, treatment with MARK (Figure 4C) or PKA (not shown) had no inhibitory effect on microtubule binding. We then generated the single mutation of S47

to alanine in a wild-type Dcx-MBP fusion protein; the S47A mutation alone completely inhibits the regulation of microtubule binding by PKA (Figure 4C). Thus, we conclude that the S47 residue is crucial for the negative regulation of Dcx's microtubule affinity.

Phosphorylated Substrates of MARK Are Present in a Complementary Pattern to Dcx Localization in Neurons

We have so far shown that Dcx localizes to microtubules in the tips of leading processes and growing neurites, that inhibiting PP2A both increases the extent of Dcx phosphorylation and displaces Dcx from neuronal growth cones, and that in vitro phosphorylation by MARK or PKA of Dcx at a serine residue (one of which is a target of missense mutations in X-LIS) negatively regulates the interaction of Dcx with microtubules. We hypothesize that Dcx is maintained at the tips of neurites by phosphatase activity and is prevented from associating with microtubules along the neurite shaft by a kinase (i.e., MARK). This hypothesis predicts that MARK/PKA kinase activity should form a pattern complementary to that of Dcx in neurons.

Because the 12E8 antibody recognizes the phospho-specific form of the KIGS motif, a common target of MARK and PKA phosphorylation, 12E8 was used as a probe to reveal indirectly kinase activity in situ. Neurons were fixed in methanol to extract soluble proteins and then immunostained for both Dcx and 12E8. This consistently generated a complementary staining pattern characterized by an abrupt transition from 12E8 immunoreactivity (inferred kinase activity) and Dcx signal at neurite tips of cortical neurons (Figures 5A–5C) and leading processes in migrating SVZa neurons (Figures 5D and 5E). Neurons treated under conditions shown previously to increase MAP phosphorylation (Okadaic acid) (Mawal-Dewan et al., 1994) or Tau dephosphorylation (pervanadate) (Mandell and Banker, 1998) show a corresponding increase and decrease, respectively, in the strength of 12E8 immunoreactivity visible in Western blots, corresponding to changes in phosphorylation status under these conditions (Figure 5F). In addition, de-
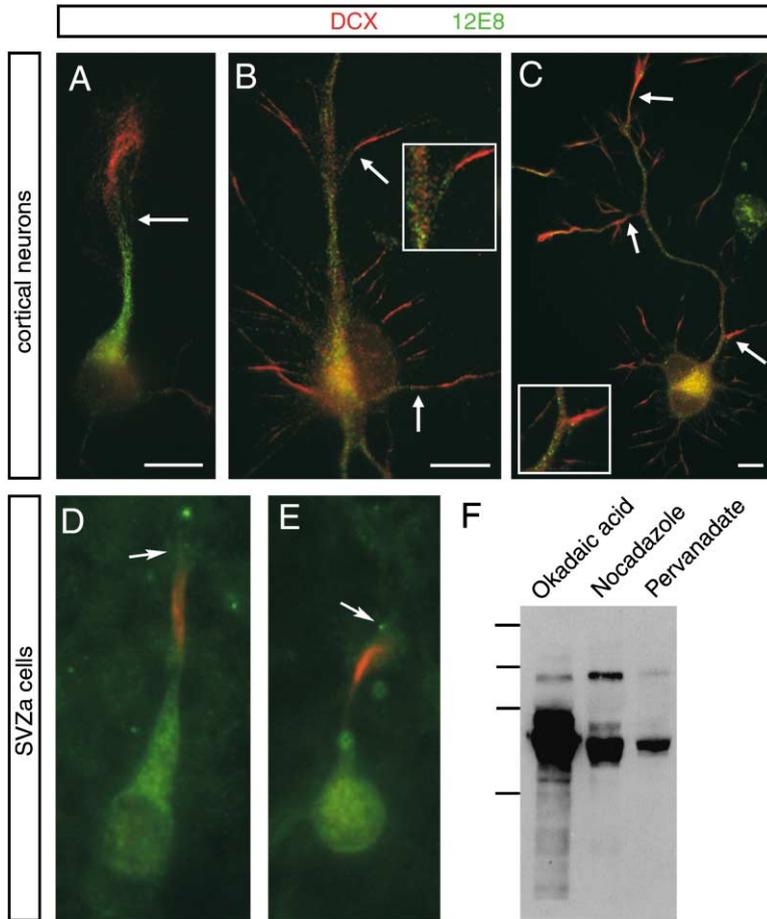
Figure 5. Complementary Patterns of Doublecortin Immunoreactivity and Kinase Activity in Neurites and in Migrating Neurons

(A–C) Spatial segregation of Dcx (red) and 12E8 immunoreactivity (green) in cortical neurons cultured on polylysine. Labeling patterns with the two antibodies were nearly completely complementary at the distal regions of neurites, with Dcx concentrated at neurite tips and abrupt transitions into regions of 12E8 immunoreactivity (arrows). Scale bars equal 10 μm. The insets in (B) and (C) show higher magnification views of the regions marked by the top arrow in (B) and the bottom arrow in (C). In cellular domains where both Dcx and 12E8 staining are present, the two antibodies appear to label non-overlapping domains.

(D and E) Spatial segregation of Dcx (red) and 12E8 immunoreactivity (green) in SVZa cells migrating in Matrigel. Note that the distal edges of the leading processes (arrows) lack Dcx. Scale bar equals 10 μm.

(F) Western blot analysis of cultured cortical neurons treated with Okadaic acid, nocodazole, or pervanadate, treatments known to influence the phosphorylation state of Tau. The 12E8 antibody recognizes a number of proteins in addition to Tau. Size standards are 218, 107, 67, and 44 kDa.

stabilization of microtubules by nocodazole altered 12E8 immunoreactivity, leading to a decrease in labeling relative to Okadaic acid treatment (Figure 5F). Although Tau is probably the most prominent protein recognized by 12E8, numerous proteins are clearly recognized by this antibody.

MARK Localizes to Membranes in Neurons

To visualize the distribution of MARK in neurons, rabbit polyclonal antibodies were raised to a peptide containing sixteen amino acids of the amino terminus of MARK2. These antibodies recognize a single band of ~90 kDa when applied to a Western blot of embryonic cortex (Figure 6D) or bacterially produced MARK2 (Figure 6E). Because the peptide sequence used to produce this antiserum is common to all members of the MARK family, we cannot ascertain definitively which MARK isoform is expressed by cortical neurons. In agreement with previous work on transfected tissue culture cells and N2 neuroblastoma cells (Biernat et al., 2002), MARK immunostaining appears to localize to the subcortical actin network and/or is associated with the cell membrane. MARK immunoreactivity decorates the entire cell surface, from soma to growth cone, suggesting that kinase activity has the potential to be deployed broadly within the neuron.

Discussion

DCX was first identified as a gene mutated in human lissencephaly (Gleeson et al., 1998; des Portes et al., 1998), and subsequent studies revealed that Dcx functions as a novel microtubule binding protein (Francis et al., 1999; Gleeson et al., 1999). We have shown here that Dcx is enriched in the leading processes of migrating neurons and that this subcellular localization is maintained by a balance of phosphatase and kinase activities. The microtubule binding activity of Dcx is subject to dynamic regulation as a result of phosphorylation of the N-terminal microtubule binding domain by the serine-threonine kinases MARK2 and PKA. Finally, we identified a serine residue that is required for the regulation of Dcx microtubule binding by these kinases in vitro and find that this serine is a target of missense mutations in X-LIS patients. These studies suggest strongly that the phosphoregulation of Dcx is crucial for its function in neuronal migration.

Neuronal Migration and the Microtubule Cytoskeleton

The migration of young neurons to their final destinations within the brain requires that cells integrate extracellular guidance cues with the intracellular mechanisms that generate and direct cell motility. Time-lapse im-
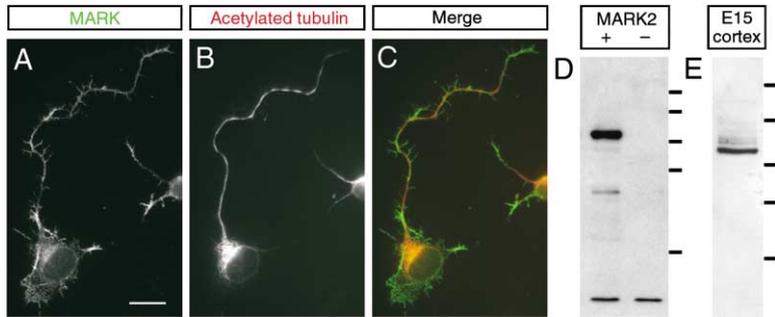
Figure 6. Localization of MARK in Cultured Cortical Neurons

(A–C) Localization of MARK in cultured cortical neurons. Embryonic cortical neurons were cultured on polylysine and stained with affinity-purified rabbit polyclonal sera raised against the amino terminus of MARK2.
(A) MARK immunoreactivity decorates the entire neuronal surface, including small filopodial processes.
(B) Immunostaining of the same cell with antibodies that recognize acetylated tubulin.
(C) Merged view of MARK (green) and tubulin (red).

(D and E) Western blots using anti-MARK sera.
(D) Left: lysate of bacteria expressing murine MARK2 (EMK). Right: vector only lysate.
(E) Blot of E15 rat cortex reveals a single prominent band of MARK immunolabeling. Markers in (D) and (E) equal 176, 114, 81, and 64 kDa.

aging studies of migrating neurons have revealed that the movement of migrating neurons is saltatory, comprised of starts and stops (Edmondson and Hatten, 1987; O'Rourke et al., 1992; Wichterle et al., 1997). During migration, the leading process can lengthen dramatically while the cell soma remains stationary. Somal movement then occurs in a saltatory manner, with the cell body translocating rapidly and abruptly to catch up with the leading process (Edmondson and Hatten, 1987; O'Rourke et al., 1992; Wichterle et al., 1997). The localization of the insoluble pool of Dcx within migrating neurons suggests a role for this protein in regulating the microtubule cytoskeleton within the leading process, where microtubules may play a direct role in governing elongation and the direction of movement. It is likely that, just as in axonal growth cones, the behavior of microtubules in the leading processes is regulated actively to enable both the growth and depolymerization of microtubules in response to extrinsic guidance cues. The modulation of Dcx microtubule binding affinity by MARK/PAR-1 and PKA kinase activity could provide a dynamic control over microtubule stabilization and bundling in the leading process, thus enabling young neurons to migrate within the complex environment of the developing brain. A key residue for the regulation of Dcx binding affinity by these kinases, S47, is the site of X-LIS patient missense mutations (Kim et al., 2003; des Portes et al., 1998). These observations provide powerful evidence that regulatory interactions between serine-threonine kinases and Dcx play a critical role in enabling Dcx to function appropriately as a MAP during normal migration.

Regulation of the Microtubule Binding Affinity of Dcx by MARK and PKA Kinase Activity

It is clear from patient missense mutations that S47 is an important residue for Dcx function in vivo. We suggest that this residue is important for the spatial control of Dcx's microtubule binding activity. Both S47 and the "canonical" KXGS MARK site at S115 are found within the first Dcx repeat. Recent structural studies of this domain have found that it binds preferentially to polymerized tubulin and not tubulin dimers (Kim et al., 2003). The second Dcx repeat is less structured and can bind tubulin dimers as well as to microtubule polymers (Kim et al., 2003). The spatial control of S47 phosphorylation

may provide a means by which to target Dcx that is already bound to tubulin dimers to the growing regions of microtubule polymers at the leading edge of migrating neurons (Kim et al., 2003; Moores et al., 2003). However, this model remains to be tested directly.

We focused on putative phosphorylation sites present in the microtubule binding domains of Dcx, as these represent likely sites for regulation based on studies of the binding affinities of the Dcx domains and those of other MAPs (Drewes and Mandelkow, 1998). We have utilized a candidate-based approach to understanding the regulation of Dcx in vivo, and our studies do not exclude other possible kinases from a role in Dcx regulation (see the accompanying paper by Tanaka et al.). The MARK consensus phosphorylation site KXGS is present in a number of MAPs within their microtubule interaction domains, where it plays an important role in the ability of MARK to regulate microtubule binding. Although the binding of Dcx to microtubules is abrogated by MARK, and although the KIGS site within the first Dcx repeat is indeed phosphorylated by both MARK and PKA, we found that this site is not essential for regulation of microtubule binding. Instead, the critical site at S47 represents a novel substrate for MARK phosphorylation. The presence of a novel site may reflect the fact that the structure of Dcx, which resembles a GTP exchange factor, differs markedly from that of other MAPs that are regulated at KXGS sequences (Kim et al., 2003).

Our candidate approach also revealed that PKA regulates the binding of Dcx to microtubules in vitro. Like MARK, PKA phosphorylates both the KIGS site and S47 in Dcx, in addition to a number of other sites that appear not to play a critical role in the regulation of microtubule binding. The relative affinities of MARK and PKA for Dcx as a substrate are not yet known; however, in the case of Tau, it is clear that MARK shows a higher affinity for KXGS sequences than does PKA. It is not yet clear whether PKA or MARK shows a greater affinity for S47 as a substrate.

Regulation of Kinase and Phosphatase Activity in Migrating Neurons

Pharmacological studies of growth cone motility have shown that microtubule dynamics are crucial for motility. Microtubule dynamics include not only the stabilization of tubulin polymers, but also their depolymerization;

thus, Dcx presumably functions in vivo with other factors that destabilize microtubules. Catastrophe-inducing factors such as the OP18 family of proteins are present in the growth cone (Gavet et al., 2002). The antagonistic functions of Dcx and catastrophe factors, in combination with a kinase-based regulatory system, is similar in concept to the regulation of microtubules in the mitotic spindle, which is generated through the balanced activities of stabilizing MAPs, catastrophe factors, and microtubule motors (Cassimeris, 1999; Kinoshita et al., 2001). The activity and localization of these components are controlled by the kinase-based cell cycle engine (Cassimeris, 1999). Our data suggest that kinases are also involved in coordinating microtubule dynamics during neuronal migration. However, in this case kinase activity is likely to be governed by external signals such as cell adhesion molecules and chemoattractants and their downstream signaling components. While kinase activity can inhibit microtubule binding by Dcx, the results from exposing cortical neurons briefly to Okadaic acid, which selectively inhibits PP2A under the conditions used, highlight the fact that phosphatase activity also plays an important role in regulating Dcx binding and localization. How PP2A activity is modulated in vivo during cell migration remains an open question. It is thought that the catalytic subunit of PP2A acquires specificity by associating with a subunit that targets the enzyme to various substrates. Interestingly, PP2A isoforms bind both to Tau and directly to tubulin in biochemical experiments and colocalize with microtubules (Merrick et al., 1996; Sontag et al., 1995, 1999).

The activity of kinases within migrating neurons and growth cones is likely to be modulated by second messenger systems, including those downstream of receptors that respond to extracellular guidance cues. PKA is activated by cyclic AMP, which modulates growth cone turning responses to Netrin-1, ACh, MAG, and BNDF in *Xenopus* spinal neurons (Ming et al., 1997; Song et al., 1997; Sun and Poo, 2001). The actions of PKA cover a broad territory, ranging from sorting events for membrane receptors to gene regulation to the regulation of the actin cytoskeleton by phosphorylation of the Ena/VASP family of proteins (Lambrechts et al., 2000). We do not yet know what events may control cAMP levels during neuronal migration. Even less is known about the regulation of the MARK family of serine/threonine kinases, although biochemical experiments indicate that MARK proteins are activated upon phosphorylation by an unidentified kinase (Biernat et al., 2002; Drewes et al., 1997).

MARK Proteins in Development and Migration

The brains of MARK2 knockout mice have not yet been examined in detail, although these mice are subject to dwarfism, infertility, and autoimmunity later in life (Bessone et al., 1999; Hurov et al., 2001). Given that *Dcx* knockout mice show no cortical migration defects, it might be surprising if a cortical neuron migration phenotype were observed in MARK2 knockout mice, even if the primary function of MARK2 was to regulate Dcx (Corbo et al., 2002). In addition, at least two other kinases (PKA and Cdk5) can regulate microtubule binding by Dcx, suggesting that there may be redundant functions

among these kinases in vivo. Nevertheless, recent studies of MARK function in neuronal cell lines have revealed an essential role for MARK in the establishment of neuronal polarity and neurite extension, consistent with an important role for MARK in polarized growth and cell movement (Biernat et al., 2002; Nelson and Grindstaff, 1997).

MARK is positioned in an ideal location, at or near the plasma membrane, to transmit polarity information to Dcx. MARK plays a prominent role in regulating the polarity of both epithelial cells and neurons (Biernat et al., 2002; Bohm et al., 1997). Moreover, the MARK homologs PAR-1 in *C. elegans* and dPAR-1 in *Drosophila* also play critical roles in the regulation of cell polarity and polarized cell behaviors (such as asymmetric cell divisions) in the early embryo (Guo and Kemphues, 1995; Shulman et al., 2000). The targets and mechanisms of MARK functions in epithelial and embryonic polarity are not yet known. However, in neuroblastoma cells (arguably the closest system to neuronal migration in which a MARK protein has been studied), MARK2 is essential for Tau-dependent neurite formation and the emergence of polarized processes during differentiation (Biernat et al., 2002). Our studies provide support for the notion that cell polarity cues deploy MARK, and possibly other kinases such as PKA, during neuronal migration to govern the subcellular localization and microtubule binding affinity of Dcx. The dynamic regulation of Dcx within the leading process enables this protein to bind selectively to microtubules that are positioned to play key roles in motility and guidance decisions. Consistent with our study of Dcx in the leading process of migrating neurons, Khollmanskikh et al. (2003) have recently shown that the in vitro migration defect seen in mouse *Lis1* heterozygous cerebellar neurons can be rescued by pharmacological inhibition of p160ROCK. This treatment also restores filipodial number and leading edge surface area, two aspects of cell morphology that are deficient in the $Lis1^{+/-}$ neurons. In addition, the affinity of Dcx for the $\mu1$ subunit of the clathrin adaptor complex and its ability to bind the phosphorylated cytoplasmic domain of the transmembrane adhesion molecule neurofascin suggest that Dcx may also play a key role in membrane trafficking events that occur within the leading process (Friocourt et al., 2001; Kizhatil et al., 2002). Thus, we propose that the cortical migration defect observed in X-linked lissencephaly is due to defects in persistent and polarized motility, in which Dcx plays a pivotal role during neuronal migration.

Experimental Procedures

Neuronal Culture

For culture of cortical neurons, cortices from two embryonic day (E) 15 rats (Sprague-Dawley) were harvested in Ca^{2+}/Mg^{2+}-free PBS and the overlying pia was removed. Tissue was dissociated in 300 μl 0.25% Trypsin, 0.53 mM EDTA, 0.4 mg/ml DNase (Sigma) for <5 min at 37°C. Cells were triturated then resuspended in Neurobasal supplemented with glucose, B-27 salts, and Penicillin/Streptomycin/Glutamine (Invitrogen). 50 μl of cell suspension was plated onto glass coverslips or 4-well slide chambers coated with 50 mg/ml laminin or 0.25 mg/ml poly-D-lysine (Becton-Dickinson). Cells were allowed to attach and then media was changed to fresh supplemented Neurobasal. Cortical neurons were cultured 2–3 days prior to experiments. SVZa neurons were isolated from postnatal day (P)

0–2 rats. Brains were embedded in low melt agarose (Invitrogen) and sagittal sections were made using a vibratome at 250 μm. Medial sections were used to isolate anterior SVZ tissue, which was embedded in growth factor-reduced Matrigel (Becton-Dickinson) on poly-D-lysine coated coverslips. Neurons were cultured overnight and processed for immunofluorescence the next day.

Immunohistochemistry

Cortical neuron cultures were pre-extracted and fixed as in Brown et al. (1993). After rehydration in PBS, specimens were blocked in 15% normal goat serum, 1 mg/ml BSA, 0.3% Triton X-100 in PBS for 15 min in a 37°C humidified chamber (as were all subsequent antibody incubations). Peptide-directed anti-Dcx rabbit sera was used at 1:1000 and counterstained with a monoclonal antibody against tubulin (DMA1, Sigma) at 1:300. Samples were incubated with primary antibodies diluted in blocking buffer then washed in PBS. Samples were incubated in Cy2-conjugated goat anti-rabbit and Texas red-conjugated donkey anti-mouse secondary antibodies at 1:200 dilution in blocking buffer (Jackson Labs) and then washed as before in PBS. Samples were mounted in Vecta Shield (Vector Laboratories). Anti-MARK sera were raised against the peptide MSSARTPLPTLNERDC. The carboxyl terminus cysteine was added in order to couple the peptide to KLH for immunization of rabbits and to couple to Sulpholink (Pierce) resin for affinity purification.

SVZa explants were fixed in −20°C methanol, rehydrated in PBS, then immunostained as described above with anti-Dcx sera and anti-β-catenin (Santa Cruz Biotechnology) or 12E8 (gift of Peter Seubert, Elan Pharmaceuticals). The 12E8 epitopes were found to be sensitive to paraformaldehyde and phosphatases. Cultured neurons or SVZa explants were rinsed briefly in PBS containing a phosphatase inhibitor cocktail (Sigma), then fixed in −20°C methanol. All subsequent incubations contain the same phosphatase inhibitor cocktail. The 12E8 antibody was used at 1:500 dilution and Dcx antibody at 1:1000. Secondary antibodies were Alexa 488 donkey anti-mouse (Molecular Probes) and Texas red goat anti-rabbit (Jackson Labs).

Cells were viewed using a Nikon Optiphot microscope. Images were captured with a Princeton Instruments cooled CCD camera using Openlab software. Images were processed using Adobe PhotoShop 5.0.

Pharmacological Treatment of Neuronal Cultures

For Okadaic acid treatment of cortical neurons, medium was replaced with 37°C supplemented Neurobasal medium that contained either 10 nM Okadaic acid (Calbiochem), 5 μg/ml Nocodazole (Calbiochem), or 0.4% DMSO (vehicle control) at various time points. At the end of drug treatment, cells were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), rinsed in PBS, then processed for immunofluorescence. For Western analysis, 100 μg of protein from each treatment was run on 10% SDS-PAGE. Proteins were electrophoretically transferred to PVDF membrane (Immobilon) and the membrane was blocked in 5% non-fat dry milk (NFDM) in PBS for 30 min and probed with a 1:2000 dilution of anti-Dcx sera for 1 hr in the same buffer. After washing, the blot was incubated in anti-rabbit horseradish peroxidase conjugated secondary antibody (Jackson Labs) at 1:1000 in blocking buffer. The blot was washed again and developed using the ECL detection system (Amersham Pharmacia Biotech).

Protein Expression and Purification

Dcx constructs were subcloned into the bacterial expression vector pMAL-C2 (New England Biolabs) using standard methods. Oligo-directed mutagenesis was used to introduce alanine substitutions with Platinum *Pfx* Polymerase (Invitrogen). Constructs were sequenced using the ABI Big Dye kit and an ABI 3700 sequencer. Constructs were transformed into *E. coli* strain BL21DE3 BLysS for protein production. Bacterial cultures were grown in Luria Broth to log phase and then induced at 25°C for 4 hr with 0.1 mM IPTG. Cultures were pelleted, resuspended in PBS supplemented with protease inhibitors (Sigma), transferred to a microfuge tube, and kept on ice for all subsequent steps. Lysozyme was added to 0.5 mg/ml and incubated 30 min. Extract was then frozen on dry ice

and thawed to an ice slurry at 37°C, and DTT (5 mM) and Triton-X-100 (0.5%) were added. The sample was sonicated to complete lysis and shear DNA, microfuged, then transferred to a clean microfuge tube containing 50 μl of amylose resin rinsed with PBS and protease inhibitors. MBP-Dcx fusion proteins were bound to the fusion protein on ice and vortexed. Resin was pelleted in a microfuge and washed with PBS with protease inhibitors. The fusion proteins were eluted in 10 mM maltose in PBS and protease inhibitors. The resin was pelleted, the supernatant was concentrated using Microcon-30 (Millipore), and the protein was snap frozen in liquid nitrogen and stored at −80°C. We experienced proteolysis in the production of the wild-type and KIGA fusion proteins, resulting in a faster migrating band 2–3 kDa lighter than the full-length protein. This clipped protein bound microtubules in a manner indistinguishable compared to the full-length MBP-Dcx fusion protein. Only full-length unproteolyzed MBP-fusion protein was used for subsequent analysis by mass spectroscopy.

The cloning of the *Xenopus laevis* homolog of MARK will be described elsewhere. For expression of recombinant XMARK the Bac-to-bac baculovirus expression system (Invitrogen) was used. Full-length XMARK was expressed as a fusion protein with glutathione-S-transferase, separated by a PreScission protease cleavage site. Hi5 insect cells were used to express the GST-XMARK. Infected cells were harvested and resuspended in lysis buffer (50 mM Tris [pH 8.0], 5 mM EGTA, 100 mM NaF, 1mM NaVO₄, 1 mM PMSF, 1 mM Benzamidine, 1 mM DTT, 40 mM β-glycerolsphate, 10 mg/ml protease inhibitors [leupeptin, pepstatin, chymostatin], 0.1% Brij-35). Cell suspensions were sonicated and spun at 14 rpm. Supernatant was incubated with Glutathione Sepharose-4B beads (Amersham Pharmacia). The beads were washed in cleavage buffer (25 mM Tris [pH 7.5], 1 mM EGTA, 1 mM DTT, 0.03% Brij-35), then incubated with PreScission protease (Amersham Pharmacia) in cleavage buffer at 4°C overnight. After cleavage from GST, the supernatant was collected, made 10% glycerol, and snap-frozen in liquid N₂.

Kinase Reactions and Microtubule Cosedimentation

4 μg of each fusion protein was phosphorylated in a 40 μl reaction volume for 2 hr at 37°C. For MARK the reaction conditions were: 1 μg of kinase, 1 mM ATP, 40 mM HEPES (pH 7.6), 5 mM MgCl₂, 2 mM EGTA (pH 8), and 1 mM DTT and protease inhibitors. PKA (New England Biolabs) reactions were done using 10 units of kinase in 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM ATP, and protease inhibitors. Kinase reactions were centrifuged at 80,000 rpm for 30 min at 22°C in a Beckman TLA 100.2 rotor prior to microtubule cosedimentation assay. Pure bovine brain tubulin (Cytoskeleton) was polymerized in an 80 μl reaction: 1 mg/ml tubulin (80 mM PIPES [pH 6.8], 0.5 mM MgCl₂, 1 mM GTP, 1 mM EGTA), 10 μM Taxol, and protease inhibitors for 15 min at 37°C. Polymerized tubulin was pelleted at 50 Krpm for 30 min at 22°C. Taxol-stabilized microtubules were resuspended in 40 μl PEM-G and taxol. 10 μl of microtubules were added to the kinased fusion protein reaction and incubated for 15 min at 37°C. This mixture was centrifuged at 40 Krpm for 30 min at 22°C. After centrifugation, the supernatants were removed and made 1× in sample buffer and the pellets were resuspended in an equal volume of sample buffer. 20 μl of each fraction was run on 10% SDS-PAGE and stained with Coomassie. Results shown are representative of at least three independent experiments. To test for 12E8 immunoreactivity, a 10 μl aliquot of the kinase reactions (and no kinase control) were removed. As a positive control for 12E8 immunoreactivity, 1.5 μg of Tau was phosphorylated under the same conditions.

Coomassie-stained bands of both PKA and MARK kinased KIGA MBP-Dcx were excised from the lanes containing the supernatant of a microtubule cosedimentation assay. As a control, the band of nonphosphorylated wild-type MBP-Dcx pellet fraction was also excised. Gel slices were soaked overnight in water and then trypsin digested. The tryptic digests were subjected to mass spectroscopy using the Voyager-DE RP Biospectrometer.

References

Angevine, J.B., and Sidman, R.L. (1961). Autoradiographic study of
the cell migration during the histogenesis of the cerebral cortex in
the mouse. Nature 192, 766–768.

Bessone, S., Vidal, F., Bouc, Y.L., Epelbaum, J., Bluet-Pajot, M.T.,
and Darmon, M. (1999). EMK protein kinase-null mice: dwarfism
and hypofertility associated with alterations in the somatotrope and
prolactin pathways. Dev. Biol. 214, 87–101.

Biernat, J., Wu, T.Z., Timm, T., Zheng-Fischhofer, Q., Mandelkow,
E., Meijer, L., and Meijer, E.M. (2002). Protein kinase MARK/PAR-1
is required for neurite outgrowth and establishment of neuronal
polarity. Mol. Biol. Cell 13, 4013–4028.

Bohm, H., Brinkmann, V., Drab, M., Henske, A., and Kurzchalia, T.V.
(1997). Mammalian homologues of C. elegans PAR-1 are asymmetri-
cally localized in epithelial cells and may influence their polarity.
Curr. Biol. 7, 603–606.

Brown, A., Li, Y., Slaughter, T., and Black, M.M. (1993). Composite
microtubules of the axon: quantitative analysis of tyrosinated and
acetylated tubulin along individual axonal microtubules. J. Cell Sci.
104, 339–352.

Cassimeris, L. (1999). Accessory protein regulation of microtubule
dynamics throughout the cell cycle. Curr. Opin. Cell Biol. 11,
134–141.

Challacombe, J.F., Snow, D.M., and Letourneau, P.C. (1997). Dy-
namic microtubule ends are required for growth cone turning to
avoid an inhibitory guidance cue. J. Neurosci. 17, 3085–3095.

Corbo, J.C., Deuel, T.A., Long, J.M., LaPorte, P., Tsai, E., Wynshaw-
Boris, A., and Walsh, C.A. (2002). Doublecortin is required in mice
for lamination of the hippocampus but not the neocortex. J. Neu-
rosci. 22, 7548–7557.

des Portes, V., Pinard, J.M., Billuart, P., Vinet, M.C., Koulakoff, A.,
Carrie, A., Gelot, A., Dupuis, E., Motte, J., Berwald-Netter, Y., et al.
(1998). A novel CNS gene required for neuronal migration and in-
volved in X-linked subcortical laminar heterotopia and lissencephaly
syndrome. Cell 92, 51–61.

Drewes, E.B., and Mandelkow, E.M. (1998). MAPs, MARKs and mi-
crotubule dynamics. Trends Biochem. Sci. 23, 307–311.

Drewes, E.B., and Mandelkow, E. (1999). Phosphorylation of MAP2c
and MAP4 by MARK kinases leads to the destabilization of microtu-
bules in cells. Cell Motil. Cytoskeleton 44, 209–224.

Drewes, E.A., Preuss, U., Mandelkow, E.M., and Mandelkow, E.
(1997). MARK, a novel family of protein kinases that phosphorylate
microtubule-associated proteins and trigger microtubule disruption.
Cell 89, 297–308.

Edmondson, J.C., and Hatten, M.E. (1987). Glial-guided granule neu-
ron migration in vitro: a high-resolution time-lapse video micro-
scopic stud. J. Neurosci. 7, 1928–1934.

Faulkner, N.E., Dujardin, D.L., Tai, C.Y., Vaughan, K.T., O'Connell,
C.B., Wang, Y., and Vallee, R.B. (2000). A role for the lissencephaly
gene LIS1 in mitosis and cytoplasmic dynein function. Nat. Cell Biol.
2, 784–791.

Feng, Y., and Walsh, C.A. (2001). Protein-protein interactions, cy-
toskeletal regulation and neuronal migration. Nat. Rev. Neurosci.
2, 408–416.

Francis, F., Koulakoff, A., Boucher, D., Chafey, P., Schaar, B., Vinet,
M.C., Friocourt, G., McDonnell, N., Reiner, O., Kahn, A., et al. (1999).

Doublecortin is a developmentally regulated, microtubule-associ-
ated protein expressed in migrating and differentiating neurons.
Neuron 23, 247–256.

Friocourt, G., Chafey, P., Billuart, P., Koulakoff, A., Vinet, M.C.,
Schaar, B.T., McConnell, S.K., Francis, F., and Chelly, J. (2001).
Doublecortin interacts with mu subunits of clathrin adaptor com-
plexes in the developing nervous system. Mol. Cell. Neurosci. 18,
307–319.

Gavet, O., Messari, S.E., Ozon, S., and Sobel, A. (2002). Regulation
and subcellular localization of the microtubule-destabilizing stath-
min family phosphoproteins in cortical neuron. J. Neurosci. Res.
68, 535–550.

Gleeson, J.G. (2000). Classical lissencephaly and double cortex
(subcortical band heterotopia): LIS1 and doublecortin. Curr. Opin.
Neurol. 13, 121–125.

Gleeson, J.G., Allen, K.M., Fox, J.W., Lamperti, E.D., Berkovic, S.,
Scheffer, I., Cooper, E.C., Dobyns, W.B., Minnerath, S.R., Ross, M.E.,
and Walsh, C.A. (1998). Doublecortin, a brain-specific gene mutated
in human X-linked lissencephaly and double cortex syndrome, en-
codes a putative signaling protein. Cell 92, 63–72.

Gleeson, J.G., Lin, P.T., Flanagan, L.A., and Walsh, C.A. (1999).
Doublecortin is a microtubule-associated protein and is expressed
widely by migrating neurons. Neuron 23, 257–271.

Guo, S., and Kemphues, K.J. (1995). par-1, a gene required for
establishing polarity in C. elegans embryos, encodes a putative Ser/
Thr kinase that is asymmetrically distributed. Cell 81, 611–620.

Hurov, J.B., Stappenbeck, T.S., Zmasek, C.M., White, L.S., Ranga-
nath, S.H., Russell, J.H., Chan, A.C., Murphy, K.M., and Piwnica-
Worms, H. (2001). Immune system dysfunction and autoimmune
disease in mice lacking Emk (Par-1) protein kinase. Mol. Cell. Biol.
21, 3206–3219.

Illenberger, S., Drewes, G., Trinczek, B., Biernat, J., Meyer, H.E.,
Olmsted, J.B., Mandelkow, E.M., and Mandelkow, E. (1996). Phos-
phorylation of microtubule-associated proteins MAP2 and MAP4 by
the protein kinase p110mark. Phosphorylation sites and regulation
of microtubule dynamics. J. Biol. Chem. 271, 10834–10843.

Khollmanskikh, S.S., Dobrin, J.S., Wynshaw-Boris, A., Letourneau,
P.C., and Ross, M.E. (2003). Disregulated Rho GTPases and actin
cytoskeleton contribute to the migration defect in Lis1-deficient
neurons. J. Neuroscience 23, 8673–8681.

Kim, M.H., Cierpicki, T., Derewenda, U., Krowarsch, D., Feng, Y.,
Devedjiev, V., Dauter, Z., Walsh, C.A., Otlewski, J., Bushweller, J.H.,
and Derewenda, Z.S. (2003). The DCX-domain tandems of dou-
blecortin and doublecortin-like kinase. Nat. Struct. Biol. 10, 324–333.

Kinoshita, K., Arnal, I., Desai, A., Dreschel, D.N., and Hyman, A.A.
(2001). Reconstitution of physiological microtubule dynamics using
purified components. Science 294, 1340–1343.

Kizhatil, K., Wu, Y.X., Sen, A., and Bennett, V. (2002). A new activity
of doublecortin in recognition of the phospho-FIGQY tyrosine in the
cytoplasmic domain of neurofascin. J. Neurosci. 15, 7948–7958.

Lambrechts, A., Kwiatkowski, A.V., Lanier, L.M., Bear, J.E., Vande-
kerckhove, J., Ampe, C., and Gertler, F.B. (2000). cAMP-dependent
protein kinase phosphorylation of EVL, a Mena/VASP relative, regu-
lates its interaction with actin and SH3 domains. J. Biol. Chem.
275, 36143–36151.

Lin, C.H., Thompson, C.A., and Forscher, P. (1994). Cytoskeletal
reorganization underlying growth cone motility. Curr. Opin. Neuro-
biol. 4, 640–647.

Mandell, J.W., and Banker, G.A. (1998). Selective blockade of axono-
genesis in cultured hippocampal neurons by the tyrosine phospha-
tase inhibitor orthovanadate. J. Neurobiol. 35, 17–28.

Mawal-Dewan, M., Henley, J., Van de Voorde, A., Trojanowski, J.Q.,
and Lee, V.M. (1994). The phosphorylation state of tau in the devel-
oping rat brain is regulated by phosphoprotein phosphatases. J.
Biol. Chem. 269, 30981–30987.

McConnell, S.K. (1995). Constructing the cerebral cortex: neurogen-
esis and fate determination. Neuron 15, 761–768.

Merrick, S.E., Demoise, D.C., and Lee, V.M. (1996). Site-specific
dephosphorylation of tau protein at Ser202/Thr205 in response to

microtubule depolymerization in cultured human neurons involves protein phosphatase 2A. J. Biol. Chem. *271*, 5589–5594.

Ming, G.L., Song, H.J., Berninger, B., Holt, C.E., Tessier-Lavigne, M., and Poo, M.M. (1997). cAMP-dependent growth cone guidance by netrin-1. Neuron *19*, 1225–1235.

Moores, C.A., Francis, F., Perderiset, M., and Houdusse, A. (2003). A double-take on MAPs. Nat. Struct. Biol. *10*, 314–316.

Nelson, W.J., and Grindstaff, K.K. (1997). Cell polarity: par for the polar course. Curr. Biol. *7*, R562–R564.

O'Rourke, N.A., Dailey, M.E., Smith, S.J., and McConnell, S.K. (1992). Diverse migratory pathways in the developing cerebral cortex. Science *258*, 299–302.

Reiner, O., Carrozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W.B., Caskey, C.T., and Ledbetter, D.H. (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. Nature *364*, 717–721.

Rivas, R.J., and Hatten, M.E. (1995). Motility and cytoskeletal organization of migrating cerebellar granule neurons. J. Neurosci. *15*, 981–989.

Rochlin, M.W., Dailey, M.E., and Bridgman, P.C. (1999). Polymerizing microtubules activate site-directed F-actin assembly in nerve growth cones. Mol. Biol. Cell *10*, 2309–2327.

Rodriguez, O.C., Schaefer, A.W., Mandato, C.A., Forscher, P., Bement, W.M., and Waterman-Storer, C.M. (2003). Conserved microtubule-actin interactions in cell movement and morphogenesis. Nat. Cell Biol. *5*, 599–609.

Sapir, T., Horesh, D., Caspi, M., Atlas, R., Burgess, H.A., Wolf, S.G., Francis, F., Chelly, J., Elbaum, M., Pietrokovski, S., and Reiner, O. (2000). Doublecortin mutations cluster in evolutionarily conserved functional domains. Hum. Mol. Genet. *9*, 703–712.

Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V., Litersky, J.M., Schenk, D., Lieberburg, I., Trojanowski, J.Q., et al. (1995). Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. J. Biol. Chem. *270*, 18917–18922.

Shulman, J.M., Benton, R., and Johnston, D.S. (2000). The *Drosophila* homolog of *C. elegans* PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell *101*, 377–388.

Smith, D.S., Niethammer, M., Ayala, R., Zhou, Y., Gambello, M.J., Wynshaw-Boris, A., and Tsai, L.H. (2000). Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. Nat. Cell Biol. *2*, 767–775.

Song, H.J., Ming, G.L., and Poo, M.M. (1997). cAMP-induced switching in turning direction of nerve growth cones. Nature *388*, 275–279.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S., and Mumby, M.C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. *128*, 1131–1144.

Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White, C.L., Mumby, M.C., and Bloom, G.S. (1999). Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J. Biol. Chem. *274*, 25490–25498.

Sun, H.J., and Poo, M. (2001). The cell biology of neuronal navigation. Nat. Cell Biol. *3*, 81–88.

Tanaka, E.M., and Kirschner, M.W. (1995). The role of microtubules in growth cone turning at substrate boundaries. J. Cell Biol. *128*, 127–137.

Tanaka, E., Ho, T., and Kirschner, M.W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. J. Cell Biol. *128*, 139–155.

Tanaka, T., Serneo, F.F., Tseng, H.-C., Kulkarni, A.B., Tsai, L.-H., and Gleeson, J.G. (2004). Cdk5 phosphorylation of doublecortin Ser297 regulates its effect on neuronal migration. Neuron *41*, this issue, 215–227.

Taylor, K.R., Holzer, A.K., Bazan, J.F., Walsh, C.A., and Gleeson,

J.G. (2000). Patient mutations in doublecortin define a repeated tubulin-binding domain. J. Biol. Chem. *275*, 34442–34450.

Ward, M., McCann, C., DeWulf, M., Wu, J.Y., and Rao, Y. (2003). Distinguishing between directional guidance and motility regulation in neuronal migration. J. Neurosci. *23*, 5170–5177.

Wichterle, H., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic, glia-independent neuronal migration. Neuron *18*, 779–791.

Williamson, T., Gordon-Weeks, P.R., Schachner, M., and Taylor, J. (1996). Microtubule reorganization is obligatory for growth cone turning. Proc. Natl. Acad. Sci. USA *93*, 15221–15226.

Wu, W., Wong, K., Chen, J., Jiang, Z., Dupuis, S., Wu, J.Y., and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. Nature *400*, 331–336.

Yoshiura, K., Noda, Y., Kinoshita, A., and Niikawa, N. (2000). Colocalization of doublecortin with the microtubules: an ex vivo colocalization study of mutant doublecortin. J. Neurobiol. *43*, 132–139.

Zakharenko, S., and Popov, S. (1998). Dynamics of axonal microtubules regulate the topology of new membrane insertion into the growing neurites. J. Cell Biol. *143*, 1077–1086.