X-linked lissencephaly is a severe brain malformation affecting males. Recently it has been demonstrated that the *doublecortin* gene is implicated in this disorder. In order to study the function of Doublecortin, we analyzed the protein upon transfection of COS cells. Doublecortin was found to bind to the microtubule cytoskeleton. *In vitro* assays (using biochemical methods, DIC microscopy and electron microscopy) demonstrate that Doublecortin binds microtubules directly, stabilizes them and causes bundling. *In vivo* assays also show that Doublecortin stabilizes microtubules and causes bundling. Doublecortin is a basic protein with an isoelectric point of 10, typical of microtubule-binding proteins. However, its sequence contains no known microtubule-binding domain(s). The results obtained in this study with Doublecortin and our previous work on another lissencephaly gene (*LIS1*) emphasize the central role of regulation of microtubule dynamics and stability during neuronal morphogenesis.

INTRODUCTION

Cortical lamination in humans occurs over months of prenatal development. The final proper layer formation relies on an intricate balance between events of the cell cycle, proliferation, neuronal path finding and migration. The lissencephaly syndromes in humans involve abnormal cortical lamination and are medically categorized as neuronal migration defects (1,2). The various human lissencephalies are classified according to morphology or putative etiology (3,4). Two types of lissencephaly have been defined (5,6). In type I, also known as ‘classical lissencephaly’, the cortex consists of four layers instead of the normal six, whereas in type II, also known as ‘cobblestone lissencephaly’, the cortex is unlayered (4). The cortex in the case of lissencephaly is as yet unknown (19,20).

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has been suggested that loss of function of Doublecortin is the major cause of subcortical laminar heterotopia.

In order to study the function of Doublecortin, we have used an ectopic expression approach in non-neuronal COS cells. In these large well-spread cells it is easier to study the cytoskeleton and as they do not express Doublecortin, there is no interference from the endogenous protein. Doublecortin is a basic protein with an isoelectric point of 10, typical of microtubule-binding proteins. However, the Doublecortin sequence contains no known microtubule-binding domain(s). In this paper, we have demonstrated that Doublecortin binds to microtubules and increases their bundling and stabilization.

RESULTS

Intracellular Doublecortin

One of the key questions in understanding the function of Doublecortin concerns its intracellular localization. First, we examined the expression of Doublecortin in primary neuronal culture. These stainings revealed an intracellular localization that resembled that of microtubules (Fig. 1a–c). However, such studies are difficult to conduct in neurons because of the highly asymmetric shape and high local concentration of microtubules in protruding processes. In order to define the intracellular localization of Doublecortin, we transiently transfected COS-7 epithelial cells with a FLAG–Doublecortin construct and immunostained them with anti-FLAG antibodies. The FLAG–Doublecortin was stained as a network of fibers reaching the cell periphery; in some cells the nuclei were stained as well (Fig. 1d). The same pattern was also observed by staining with anti-Doublecortin antibodies (Fig. 2b and e). This was the first indication that Doublecortin co-localizes with cytoskeletal proteins. Double staining of transfected cells with anti-Doublecortin antibodies and with anti-α-tubulin antibodies revealed significant overlaps (Fig. 2b and e, anti-Doublecortin antibodies with the secondary antibody labeled with FITC and rhodamine, respectively; Fig. 2a and d, the corresponding cells stained with anti-α-tubulin antibodies; Fig. 2c and f, overlaps in the staining). Furthermore, GFP–Doublecortin revealed a similar fibrillar pattern in both live and fixed cells (data...
not shown). This fibrillar structure persisted (Fig. 2g) even when the actin cytoskeleton was completely disrupted by cytochalasin B treatment (shown in Fig. 2h, by staining of the cell in Fig. 2g with fluorescein–phalloidin, which binds to actin). Co-localization with the microtubule cytoskeleton was further demonstrated by disruption of the microtubules with nocodazole. Subsequent staining using both Doublecortin [using anti-FLAG antibodies (Fig. 2i) as well as anti-Doublecortin antibodies (Fig. 2j)] and tubulin revealed that the fibrillar structured staining disappeared (Fig. 2k).

Our results of overexpression experiments of Doublecortin in COS-7 cells suggested that Doublecortin may act similarly to classical microtubule-associated proteins (MAPs) and may influence microtubule stability. We used several approaches to examine this hypothesis. Stable microtubules are known to be acetylated in cells (26–28); therefore, we double stained transfected cells with anti-Doublecortin antibodies and anti-acetylated tubulin antibodies. As seen in Figure 3a–f, cells that overexpress Doublecortin are positive for acetylated tubulin fibers that are organized in a typical ‘spring’ fashion (compare Fig. 3a–c with d–f, respectively). Acetylated tubulin fibers are barely visible in non-transfected cells. Furthermore, microtubule bundles are observed in cells exhibiting high level overexpression of Doublecortin. This can be seen in the cell in Figure 1d, where some of the visualized fibers appear bundled.

Stable tubulin fibers form a typical ‘spring’ structure due to physical constraints imposed by the cortical actin network. The cortical actin polymers act as a physical barrier, preventing extension of the MAP-induced microtubule bundles beyond the cell periphery (29). When the cortical actin filaments are disrupted by cytochalasin B treatment, the MAP-induced microtubule bundles straighten and push out spike-like protrusions from the cell surface. This phenomenon was initially

Figure 2. Doublecortin co-localizes with microtubules. COS-7 cells were transfected with FLAG–Doublecortin and 48 h after transfections cells were fixed with cold methanol and double stained with anti-α-tubulin antibodies and secondary anti-mouse rhodamine (a) and anti-Doublecortin primary antibodies and secondary anti-rabbit FITC (b) or anti-α-tubulin antibodies and secondary anti-mouse FITC (d) and with anti-Doublecortin primary antibodies and secondary anti-rabbit Cy3 anti-rabbit coupled with anti-α-tubulin antibodies (e). (c and f) Co-localization of fibers is shown. (g) Cytochalasin B did not disrupt the fibers (stained with anti-FLAG antibodies). (h) This treatment completely disrupted the actin cytoskeleton (fluorescein–phalloidin staining). (i) The fibrous structured staining of Doublecortin was completely disrupted by nocodazole treatment, depicted by anti-FLAG staining. (j) Doublecortin staining is disrupted by nocodazole. (k) The disruption in microtubule staining is visualized by anti-α-tubulin antibodies.
observed in cells overexpressing MAP2 (30). Indeed, when
FLAG–Doublecortin or GFP–Doublecortin (data not shown)
overexpressing cells were treated with cytochalasin B, typical
protrusions appeared (Fig. 3g–j). It was possible to follow the
development of these protrusions in living cells using time-
lapse microscopy (Fig. 3k–m). The effect of cytochalasin B
was already visible 3 min after treatment (Fig. 3l). The above
experiments suggest that Doublecortin is a MAP. The precise
mode of interaction between Doublecortin and microtubules
was further investigated using biochemical methods.

Biochemical fractionation of exogenous expressed protein
corroborated the cytoskeletal localization of Doublecortin
which had been suggested by immunostaining. We used the
well-elaborated protocol of gentle cell extraction with non-
ionic detergent (0.5% Triton X-100) that removes lipids and
soluble proteins, leaving intact the detergent-insoluble matrix.

Figure 3. Doublecortin stabilizes microtubules. COS-7 cells were transfected with FLAG–Doublecortin and 48 h after transfections cells were fixed with cold
methanol and stained. (a–c) Cells stained with anti-Doublecortin primary antibodies and anti-rabbit FITC. (d–f) The corresponding cells were stained with anti-
acetylated tubulin and anti-mouse rhodamine. Note bundles in the cells. (f) It is possible to see low level expression of acetylated tubulin in two non-transfected
cells that are located above the transfected cells. (g–j) Transfected cells were treated with cytochalasin B and then fixed and stained. Note the delicate protrusions
extending from the cells. (g and i) Cells stained with anti-α-tubulin and anti-mouse rhodamine. (h and j) The cell in (g) or (i) stained with anti-Doublecortin anti-
bodies and anti-rabbit FITC. (k–m) Live cell microscopy of cells transfected with FLAG–Doublecortin and treated with cytochalasin B. Arrows denote the
extended protrusions. (l) Cell before addition of cytochalasin B. (m) Cell 3 min after addition of cytochalasin B. Note the protrusions. (m) Cell 45 min after addition
of cytochalasin B.
Figure 4. Doublecortin-transfected cells and recombinant Doublecortin. (a) COS-7 cells were transfected with FLAG–Doublecortin and 48 h after transfection cells were subject to detergent extraction as described in Materials and Methods. Ten micrograms of total protein were separated on 10% SDS–PAGE gels, immunoblotted and reacted with the corresponding antibodies; (upper panel) anti-α-tubulin; (second panel) anti-Doublecortin; (third panel) anti-FLAG; (lower panel) anti-acetylated tubulin. Note that mock-transfected cells do not express Doublecortin, as demonstrated both by the anti-Doublecortin and anti-FLAG antibodies. Transfected cells were treated with nucodazole. In extracts from the treated cells there is a shift of tubulin and Doublecortin from the insoluble to the soluble fraction. (b) The distribution of tubulin in the insoluble and soluble fractions of mock and transfected cells (as described above) was quantified from the results of five repeats of the experiment. (c) Microtubules were pre-assembled from purified tubulin in PEM buffer. Polymerization was done in the presence of a microtubule-stabilizing drug (Taxol), a microtubule-destabilizing drug (Nucodazole, Noc) or without addition of drugs (−). GST–Doublecortin or GST alone were added after polymerization and incubated with the microtubules for 10 min at 37°C. Assembled microtubules and associated proteins (pellet, P) were sedimented by centrifugation. Pellets and supernatants (S) containing soluble proteins were separated on SDS–PAGE gels. Gels were stained with Coomassie blue.

Doublecortin and microtubule assembly

The next issue pursued was whether Doublecortin has an effect on microtubule assembly. To determine whether Doublecortin is involved in microtubule formation, we measured the assembly rate of tubulin using a light scattering assay (Fig. 5e). This assay is based on an increase in optical density absorbance as microtubules polymerize. Addition of recombinant GST–Doublecortin increased the rate of assembly and the total amount of assembled tubulin in a dose-dependent manner far beyond those obtained with tubulin itself (Fig. 5a) or by addition of GST control protein to tubulin (shown in a similar experiment in Fig. 7e). Addition of Doublecortin also reduced the critical concentration of tubulin required for polymerization, as tested by DIC microscopy. Tubulin will polymerize without the need for external nucleation sites at specific temperature-dependent concentrations (a higher temperature will require a lower concentration of tubulin), termed the 'critical concentration' (31). We assayed whether the critical concentration of tubulin polymerization was altered by the addition of purified Doublecortin. Different tubulin concentrations were incubated at varying temperatures ranging from 26 to 38°C with GST–Doublecortin or GST alone as a control (data not shown). Addition of GST–Doublecortin (1:5 molar ratio) to tubulin (16 µM) caused a reduction of 6°C in the temperature at which initial polymerization was observed. Likewise, using 20 µM tubulin and GST–Doublecortin microtubules reduced the temperature required for initial polymerization by 4°C. These observations
Doublecortin in COS-7 cells suggested that this protein may cause microtubule bundling in vitro as measured using a light scattering assay. (Fig. 5c) Doublecortin:tubulin ratio 1:2; (d) Doublecortin:tubulin ratio 1:1. The assembly rate of tubulin was changed as indicated; the concentration of tubulin used was 16 µM. The optical absorbance was recorded every minute.

were supported by varying tubulin concentrations incubated at a fixed temperature of 26°C. The effect of overexpression of Doublecortin in COS-7 cells suggested that this protein may cause microtubule bundling. Similarly, addition of Doublecortin to tubulin resulted in microtubule bundling in vitro, clearly visualized by DIC microscopy (Fig. 5a–d). Increasing concentrations of Doublecortin were used while tubulin concentrations were held constant at 10 µM (Fig. 5b–d). While in the control there are a few visible microtubules (Fig. 5a), the number of filaments increased dramatically when Doublecortin was added (Fig. 5b–d). Clear bundles were visible when the Doublecortin:tubulin ratio was increased to 1:2 (Fig. 5c); the effect was even more striking when this ratio was increased to 1:1 (Fig. 5d).

Mapping of Doublecortin and microtubule interaction

In order to map the cytoskeletal interaction we generated several truncated fragments of Doublecortin (Fig. 6a). As computer analysis did not reveal any putative functional domains and no crystal structure is yet available for this protein, we arbitrarily divided the coding region into three domains. Both in vivo experiments using a series of FLAG–Doublecortin constructs expressed in COS cells and in vitro assembly experiments using GST-tagged protein produced in bacteria (Fig. 6) were used to delineate the interacting domain(s). COS-7 cells were transfected with the different fragments and the pattern of staining as well as biochemical fractionation were determined as have been described. It was clear that FLAG–Doublecortin EcoRV (1–213) was associated with the microtubules while FLAG–Doublecortin EcoRV–end (213–361) was soluble (Fig. 6b and c). This was demonstrated by detergent extraction where essentially all of the FLAG–Doublecortin EcoRV (1–213) was found in the insoluble fraction (Fig. 6b). Furthermore, immunostaining of FLAG–Doublecortin EcoRV (1–213) depicted a clear fibrillar structure versus the FLAG–Doublecortin EcoRV–end (213–361), which stained the cell with no fibers (Fig. 6c versus d). FLAG–Doublecortin BamHI–end (110–361) was also expressed in COS-7 cells, but did not stain microtubules (Fig. 6e).

In addition, in vitro analysis of GST–Doublecortin EcoRV (1–213) revealed direct binding to tubulin (Fig. 6g), while the fragment of GST–Doublecortin BamHI–end (110–361) demonstrated very weak binding (Fig. 6f). The GST–Doublecortin EcoRV (1–213) fragment was also tested for bundling activity. Samples with and without this Doublecortin fragment were processed for electron microscopy. Dense bundles were visible by negative staining (Fig. 7a and c). Most of the bundles appeared very dense and dark (Fig. 7a), while some were less dense (Fig. 7c). Without the addition of Doublecortin, only single microtubule filaments are visible (Fig. 7b and d). Two Doublecortin fragments, Doublecortin EcoRV (1–213) and Doublecortin BamHI–end (110–361) were tested in the light scattering assay. As clearly demonstrated in Figure 7e, while Doublecortin EcoRV (1–213) had activity very similar to the full-length Doublecortin. Doublecortin BamHI–end (110–361) activity was very low and the curve was quite similar to the measured activity of pure tubulin or to the activity of tubulin with GST control protein. This same fragment was tested for bundling activity using DIC microscopy; no bundles were apparent (data not shown). Our attempts to express shorter fragments were unsuccessful: the first third of the protein (1–110) was not expressed in mammalian cells nor in bacteria and the second third (110–213) was not expressed in mammalian cells (Fig. 6). The simplest interpretation of our results localizes the binding/bundling activity of Doublecortin to the first third of the protein. Future experiments combining structural data with mutagenesis of specific amino acids will allow us to define more accurately this interaction.

DISCUSSION

Doublecortin is a MAP

Our results suggest that Doublecortin may function as a MAP. The interaction between Doublecortin and microtubules is direct and does not require any additional mediators. This interaction results in stabilization and bundling of microtubules both in vivo and in vitro. Doublecortin is a basic protein with an isoelectric
point of 10, typical of other microtubule-binding proteins. Sequence analysis did not detect any microtubule-binding domains; however, our deletion analysis localizes the region of binding to amino acids 1–110. We believe that this function is highly significant for the role of Doublecortin during brain development. Several events involved in the formation of the structure of the cerebral cortex require the presence of microtubules. These include neuronal migration, nuclear displacement and process formation. Cell migration is characterized by dynamic interactions between the substrate and the cytoskeleton-associated motile apparatus inside the cell (32). The role of microtubules in the formation of growth cones is also well documented (33,34). The active migration of neurons from their sites of origin to their final destination requires unidirectional translocation of the nuclei and
somatic cytoplasm within the growing leading processes. It has been suggested that the dynamics of slow polymerization in combination with fast disintegration of oriented microtubules create forces that contribute to the piston-like salutatory displacement of the nucleus and cytoplasm within the membrane cylinder of the leading process of the migrating neuron (35). Indeed, microtubules are a predominant component of the neuronal cytoskeleton and comprise 15–20% of total cell protein in brain and they are essential for the development of neuronal morphology (36). The proposed role of microtubules in nuclear displacement during neuronal migration does not exclude the synergistic action of actin-like contractile proteins which may also participate in this event (37). However, it does fit with the finding that disruption of microtubule structure results in collapse of the migrating cell body and cessation of nuclear translocation (37).

The outgrowth of neuronal processes involves the interaction of two major components of the neuronal cytoskeleton, microtubules and actin filaments. These produce counteractive forces, tension generated by actin filaments of the cortical cytoskeleton (38,39) and compression produced by microtubule bundles in the cytoplasm (40,41). To be capable of supporting processes, the neuronal microtubules should have several special properties. They must be more stable than the microtubules of non-neuronal cells and they must be sufficiently stiff to support the elongate processes as they are formed. These special properties are thought to depend on a set of structural MAPs.

**Doublecortin stabilizes microtubules**

We have demonstrated here that Doublecortin stabilizes microtubules when ectopically expressed in non-neuronal cells. Microtubule stabilization was visible from the shift of tubulin to the insoluble fraction using a detergent extraction procedure, by the existence of acetylated tubulin fibers and protrusion of microtubule spikes after disruption of the cortical actin network. Furthermore, in vitro analysis demonstrated that Doublecortin reduced the critical concentration required for polymerization and caused bundling. Doublecortin is encoded by 361 amino acids that did not reveal any functional domains by computer searches. Several MAPs have been shown to bind to microtubules via short sequences with repeated amino acids motifs. However, we did not detect short repeated amino acids motifs or any clusters of charged amino acids that would imply microtubule binding. One mode of regulating Doublecortin microtubule binding may be through phosphorylation. In general, it has been shown that there is an inverse correlation between phosphorylation and microtubule binding; the more phosphorylated the protein, the less it binds to microtubules and stabilizes them (42–44). The phosphorylation state of MAPs changes during development (reviewed in refs. 34,45). This may be due to combined activities of kinases or phosphatases (46). This post-translational modification is believed to be the major mode of MAP function regulation. Within Doublecortin’s first 100 amino acids there is a putative site for c-Abl phosphorylation (20). We have preliminary results indicating that Doublecortin is phosphorylated by c-Abl (O. Reiner, unpublished data) and we are investigating how this affects Doublecortin function. The most probable interpretation of our results localizes the region of Doublecortin–microtubule interaction to the first third of this protein. Bundling requires at least two binding sites that may reside within a single full-length Doublecortin sequence or result from homodimerization of two Doublecortin molecules. We favor the first option, as our preliminary data do not demonstrate homodimerization (T. Sapir, unpublished data).

We believe that the function of Doublecortin as a stabilizer of microtubules is highly relevant to the disease phenotype. We have previously characterized the interaction between LIS1 and microtubules and its influence on microtubule dynamics (11). As mutations in *LIS1* or *doublecortin* result in lissencephaly type I it is likely that their interaction with the microtubule cytoskeletal component of neuronal cells is an important feature in the final obstruction of cortical layering. *Lis1* gene targeting in the mouse demonstrated a direct neuronal migration defect (16; O. Reiner, unpublished data). We have previously proposed that nuclear migration may be important for the neuronal migration defect (13). This hypothesis was based on defects in nuclear migration.
due to mutations of the LIS1 homolog in *A. nidulans*, NudF (14). Furthermore, in higher eukaryotes LIS1 physically interacts with gene products that are involved in the nuclear migration pathway in *A. nidulans*. This includes LIS1 interactions with tubulin (11) and with the mammalian homolog of NuDC (13). At present, we do not know whether LIS1 and Doublecortin are part of the same or different pathways. Not all lissencephaly-associated gene products will interact with microtubules. For example, a gene involved in Fukuyma-type congenital muscular dystrophy, which is associated with type II lissencephaly, was identified (47). Its protein product, Fukutin, is a secreted protein of unknown function (47); however, as a secreted protein it is not expected to interact with the cytoskeleton.

The stabilizing effect of Doublecortin on microtubules ascribes a function to this disease gene that may explain part of the pathophysiology of the disease phenotype. Furthermore, it expands our growing understanding of basic processes that are involved in cortical layer formation.

**MATERIALS AND METHODS**

**Cell culture**

COS-7 cells (48) were grown at 37°C with 5% CO₂ and 95% air in DMEM nutrient medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cultures were split using standard trypsinization procedures. COS-7 cells were transfected using the DEAE transfection procedure (49) with 20 µg of DNA in the absence of serum in the medium. Four hours after transfection the medium was aspirated and 10% DMSO was added for 2 min. The cells were washed and fresh standard growth medium added. Transfected cells were then analyzed at various times for expression of exogenous protein. Efficiency of transfections was estimated using a GFP expression vector (Clontech, Palo Alto, CA).

Primary neuronal cultures were prepared from cortex of E15.5 mouse strain MF1. The neurons were dissociated, plated on poly(ornithine)-coated coverslips and grown in culture as described (50).

**Plasmid construction**

The constructs discussed in this paper are summarized in Figure 6a. The GST fusion proteins were made in pGEX4T-1 (Pharmacia, Piscataway, NJ). The full-length clone was constructed by preparation of a 1.2 kb *HindIII-*Smal fragment, using the *HindIII* site 53 nt upstream of the start methionine and a *Smal* site present in the cDNA vector. The *HindIII* site was blunt ended and the fragment was cloned in the pGEX4T-1 *Smal* site. The construct was checked by sequencing of both ends. pGEX-BamHI-end is a subclone of a *BamHI* fragment in pGEX4T-1 digested with *BamHI*. The construct was checked by sequencing. pGEX-BamHI was prepared by digestion of a 370 bp *HindIII*-BamHI fragment blunt ended and cloned in the *Smal* site of pGEX4T-1. The construct was checked by sequencing. GST fusion proteins were produced in the BL21 lysE strain of *Escherichia coli* and purified using glutathione-agarose (Sigma, Rehovot, Israel). Proteins that were not present in soluble form in bacteria were regarded as not expressed, as it was not possible to purify the proteins in the native conformation for biological experiments. The tagged proteins for expression in mammalian cells were made by subcloning the described fragments into pECE (51) with a FLAG epitope at the N-terminus or a GFP tag using pEGFP-N1 (Clontech). For the 5’ and 3’ ends, EcoRI sites derived from construct pGEX4T-1 were used. PCR of amino acids 110–213 was carried out using 5’ primer 5’-CAGCGTGACGATCAGGATCACCAGGATCCAGGAAGATCGG-3’, introducing a new *KpnI* site, and 3’ primer MYST2 5’-CTGGTTTCTCATCCAAGTGGTAGAG-3’. The PCR product was digested with *KpnI* and EcoRV and the fragment was cloned into PECE-FLAG using *KpnI* and *Smal* sites. Amino acids 110–end were introduced into PECE-FLAG using 5’ primer 5’-CAGCGTGACGATCAGGATCACCAGGATCCAGGAAGATCGG-3’, introducing a new *KpnI* site, and the 3’ primer from the pGEX vector. The PCR product was digested with *KpnI* and EcoRI and cloned into PECE-FLAG digested with the same restriction enzymes. Plasmids with inserts derived from PCR were completely sequenced. Expression of FLAG-tagged proteins was examined by immunohistochemistry and western blot analysis.

**Antibodies**

Anti-α-tubulin (monoclonal, clone DM1A) or anti-acetylated tubulin (monoclonal, clone no. 6-11B-1) were purchased from Sigma. Anti-doublecortin polyclonal antibody J74 was produced against a mixture of peptides (52). Anti-FLAG M2 monoclonal antibody was obtained from Kodak (New Haven, CT). All secondary antibodies were from Jackson ImmunoResearch (West Grove, PA); peroxidase-conjugated affinipure goat anti-mouse IgG (H+L); lissamine/rhodamine-conjugated affinipure goat anti-mouse IgG (H+L); Cy3-conjugated affinipure goat anti-rabbit IgG (H+L); FITC-conjugated affinipure goat anti-mouse or anti-rabbit IgG (H+L).

Fluorescein–phalloidin (Molecular Probes, Eugene, OR) was used at 2 U/ml.

**Immunostaining**

Briefly, transfected cells were plated on glass coverslips. After 48 h they were washed twice with phosphate-buffered saline (PBS), then fixed and permeabilized simultaneously in cold methanol for 10 min. After fixation the cells were incubated with 30 µl of the first antibody for 60 min at room temperature, then washed three times with PBS and incubated for 30 min with 30 µl of fluorescent-conjugated secondary antibodies. The coverslips were washed three times with PBS, drained and mounted. The immunostaining was visualized using an Olympus microscope (IX50 model; Hamburg, Germany) using the appropriate filters. Photomicrography was with Kodak 160T film. For microtubule disruption, cells were treated with 5 µM nocodazole (Sigma) overnight. For actin disruption cells were treated with 20 µM cytochalasin B for 2 h (Sigma) as described before (30). Neurons (Fig. 1) were observed under a Bio-Rad (Hercules, CA) confocal microscope.

**Live-cell microscopy**

For live microscopy, COS cells were plated on coverslips glued with parafilm across holes in the bottom of Petri dishes. Transfection was done with Doublecortin and GFP plasmids in a 9:1 ratio as described. Approximately 48 h after transfection the dishes were moved to the microscope. Prewarmed mineral oil was spread across the surface of the medium to prevent evaporation and loss
of CO₂. Transfected cells were located using the GFP marker. They often had an atypical morphology and in some cases bundles of microtubules could be seen directly.

Observations were made in differential interference contrast on an inverted microscope (Zeiss Axiovert 35, Fluor 100×/1.3 objective) with red light illumination. The objective was heated electrically to 37°C (Biop techs, Butler, PA). Temperature of the cells was maintained by thermal conduction through the immersion oil. Video images were recorded by a digital CCD camera (iSight; Tirat haCarmel, Israel) onto S-VHS cassettes.

After choosing a cell, 1 ml of new medium containing cytochalasin B was introduced by syringe underneath the mineral oil. The process shown in Figure 3k−m took 50 min, following which the medium was exchanged three times for fresh medium without cytochalasin B. The prominent branches bent and retracted, some partially and others completely (data not shown).

**Detergent extraction assay**

The detergent extraction assay was performed essentially as described by Cohen et al. (53). Sub-confluent cultures of transfected COS-7 cells, grown on 9 cm plates, were washed once with PBS and then with MES buffer (50 mM MES pH 6.8, 2.5 mM EGTA, 2.5 mM MgCl₂). The cells were extracted for 3 min with 0.5 ml of 0.5% Triton X-100 in MES buffer supplemented with protease inhibitors (Sigma). The supernatant (the soluble fraction, termed Sol) was collected, centrifuged for 2 min at 16 000 g at 4°C and the clear supernatant was then transferred to new tubes. Two volumes of cold ethanol were added and the tubes were incubated at −20°C overnight, centrifuged for 10 min at 16 000 g at 4°C and resuspended in 200 µl of 2X protein sample buffer without dye. The detergent-insoluble matrix (InSol) remaining on the plate was extracted in 200 µl of 2X protein sample buffer, scraped from the plate with a rubber policeman and collected into tubes. Treatment with nocodazole was at 10 µM for 4 h prior to extraction. The samples were loaded on 10% SDS−PAGE gels; equal volumes of Sol and InSol were loaded according to the volume that contained 100 µg of protein in the soluble fraction. Proteins were detected by western blot analysis using primary anti-FLAG antibodies (1:300 dilution), antibodies against Doublecortin (1:2000 dilution), anti-tubulin antibodies (1:2000 dilution) and peroxidase-conjugated secondary antibodies (1:10 000 dilution). The chemiluminescence reaction was developed using SuperSignal substrate (Pierce, Rockford, IL).

**Microtubule assembly in vitro**

**Rate measurements.** Tubulin was purified as described (11). The assembly rate of tubulin to form polymers was monitored using a light scattering assay (54, 55). Purified tubulin was diluted in PEM buffer (100 mM PIPES pH 6.9, 1 mM MgSO₄, 1 mM EGTA) supplemented with 1 mM GTP to a final concentration of 16 µM. Recombinant proteins were dialyzed against PEM buffer prior to addition to the tubulin solution. Absorbance was measured at 350 nm at 1 min intervals in a UVic on spectrophotometer equipped with temperature controlled cells. Switching the temperature to 37°C induced assembly.

**Electron microscopy.** Samples were prepared as described for DIC microscopy using tubulin at a concentration of 15 µM with or without the addition of 9 µM EcoRv 1–213 fragment and incubated for 15 min at 37°C. Eight microliters of each sample were applied to a carbon-coated copper 400 mesh electron microscope grid, which had previously been glow-discharged to render the carbon surface hydrophilic. The grid was then rinsed with water, and 10 µl of uranyl acetate stain (1% in water) was applied. The grid was blotted after 30 s. Samples were examined with a Philips CM12 electron microscope operating at 100 kV.

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NOTE ADDED IN PROOF
A paper was published after this manuscript was accepted which describes Doublecortin as a MAP: Glees, 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.

REFERENCES