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A role of MAP1B in Reelin-dependent Neuronal Migration

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The signaling cascades governing neuronal migration are believed to link extracellular signals to cytoskeletal components. MAP1B is a neuron-specific microtubule-associated protein implicated in the control of the dynamic stability of microtubules and in the cross-talk between microtubules and actin filaments. Here we show that Reelin can induce mode I MAP1B phosphorylation, both *in vivo* and *in vitro*, through gsk3 and cdk5 activation. Additionally, mDab1 participates in the signaling cascade responsible for mode I MAP1B phosphorylation. Conversely, MAP1B-deficient mice display an abnormal structuring of the nervous system, especially in brain laminated areas, indicating a failure in neuronal migration. Therefore, we propose that Reelin can induce post-translational modifications on MAP1B that could correlate with its function in neuronal migration.

Keywords: cyclin-dependent kinase 5, glycogen synthase kinase 3, microtubule-associated protein, neuronal migration, Reelin

Introduction

Ordered neural migration is an essential step in the organization of brain nuclei and laminated brain regions. Another essential step in neural development is axonal guidance and the formation of neural connections with specific targets. These two processes are controlled by specific guidance cues, including extracellular and membrane-anchored proteins (Tessier-Lavigne and Goodman, 1996; Rice and Curran, 2001). Both in experimental animals and in humans, disruption of ordered neural migration leads to structural and functional defects that are associated with neurological abnormalities including sensory-motor disorders and mental retardation (Feng and Walsh, 2001; Rice and Curran, 2001).

Reelin is an extracellular matrix protein which is essential for the correct migration and positioning of neurons in laminated brain regions such as the cerebral cortex, hippocampus and cerebellum (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995; Ogawa *et al.*, 1995). Reelin receptors include the very low density lipoprotein receptor (VLDLR) and the ApoE receptor 2 (ApoER2) (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999). Binding of Reelin to these receptors leads to phosphorylation of the adaptor protein Disabled 1 (mDab1), which may control the activity of the serine/threonine kinase cyclin-dependent kinase 5 (CDK5) through interaction with the regulatory subunits p35 and p39 (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Howell *et al.*, 1999a,b; Walsh and Goffinet, 2000; Keshvara *et al.*, 2001; Ko *et al.*, 2001). It has recently been shown that Reelin activates the tyrosin kinases Fyn and Src to phosphorylate mDab1 (Arnaud *et al.*, 2003; Bock *et al.*, 2003), and that Reelin stimulates serine phosphorylation of glycogen synthase kinase 3 (GSK3), which is believed to decrease GSK3 activity (Beffert *et al.*, 2002). Reelin has also been reported to bind to α3 and β1 integrins, and to cadherin neuronal related (CNR) protocadherins (Senzaki *et al.*, 1999; Dulabon *et al.*, 2000). Since *dab*1(−/−) and *cdk*5(−/−) mice show migration abnormalities reminiscent of those in *reeler*, these genes are believed to be essential for Reelin signaling (Ohshima *et al.*, 1996; Howell *et al.*, 1997; Sheldon *et al.*, 1997; Gilmore *et al.*, 1998; Kwon and Tsai, 1998). Because substrates of CDK5 include cytoskeletal proteins (Ishiguro *et al.*, 1994; Pigino *et al.*, 1997), the Reelin signaling might transduce a signal that regulates the cytoskeleton and cell motility during neuronal migration, in a manner as yet unknown. Indeed, mutations in the microtubule-associated proteins (MAPs) LIS1 and Doublecortin cause severe migration disorders (Hirotsune *et al.*, 1998; Francis *et al.*, 1999; Gleeson *et al.*, 1999; Cahana *et al.*, 2001).

MAP1B is a neuron-specific MAP that is expressed in virtually all developing neurons, both *in vivo* and *in vitro* (Avila *et al.*, 1994a). Its function is regulated at the post-translational level by phosphorylation (Avila *et al.*, 1994b). Mode I phosphorylation is mediated by CDK5 and GSK3, being dynamically regulated by functional stimuli such as lysophosphatidic acid (LPA) (Sayas *et al.*, 1999), and axonal regeneration (Gonzalez-Billault *et al.*, 2004). Mode II phosphorylation is catalyzed by casein kinase II and appears to be activated constitutively (Diaz-Nido *et al.*, 1988; Gonzalez-Billault *et al.*, 2004). MAP1B has been implicated in neurite extension, the dynamic stability of microtubules and the cross-talk between microtubules and actin microfilaments, in a mode I phosphorylation-dependent manner (Lucas *et al.*, 1998; Goold *et al.*, 1999; Mack *et al.*, 2000; Gonzalez-Billault *et al.*, 2001).

*Map1*B-deficient mice have variable degrees of abnormalities in axonal tracts, which are believed to be a consequence of decreased capacity for axonal elongation in these mice (Takei *et al.*, 1997, 2000; Gonzalez-Billault *et al.*, 2000; Meixner *et al.*, 2000; Teng *et al.*, 2001). A role in axonal growth and synaptogenesis has also been proposed for the *Drosophila* MAP1B ortholog Futsch (Hummel *et al.*, 2000; Roos *et al.*, 2000). In contrast, the involvement of MAP1B in neuronal migration is more controversial (Gonzalez-Billault *et al.*, 2000; Takei *et al.*, 2000; Teng *et al.*, 2001). Here we examine whether MAP1B function can be modified by a cannonical signaling pathway controling neuronal migration, such as Reelin. We show that *map1*B mutant mice display migration deficits in several brain structures, and also that Reelin may control mode I phosphorylation of MAP1B in a GSK3- and CDK5-dependent manner. These

results suggest that Reelin could modify, through MAP1B function, the neuronal cytoskeleton of migrating neurons.

Materials and Methods

Animals

Generation of *map1*B mutants (R1/NMR1 background, 129 substrain) was made by the gene-trapping approach (Chowdhury *et al.*, 1997). The gene-trapping vector contained a fused chimeric gene composed of neomycine phosphotransferase (neo^r) and β-galactosidase (β-gal) controlled by the promoter of the endogenous trapped gene (Chowdhury *et al.*, 1997). To genotype the mutant mice, genomic DNA was isolated from mice tails and analyzed by polymerase chain reaction (PCR), using oligonucleotides corresponding to neomycine phosphotransferase gene (neo^r) contained in the gene-trapping vector (Gonzalez-Billault *et al.*, 2000). Heterozygous animals were breed to obtain homozygous *map1*B mutants. The genotype of homozygous mutants was indicated by abnormal limb posture (Gonzalez-Billault *et al.*, 2000), and subsequently confirmed by Western blot analyses of spinal cord protein extracts immunostained with anti-MAP1B (monoclonal antibody 125) and anti-β-galactosidase (Promega) antibodies. All animals were treated according to standard, internationally approved protocols.

Histology

Embryos were transcardially perfused at embryonic day (E) 18 with 4% paraformaldehyde. Dissected brains were postfixed in the same fixative, cryoprotected and frozen in dry ice. Coronal sections (40 μm thick) were Nissl-stained or immunostained with the following antibodies: anti-Calretinin antibodies (Swant, Bellinzona, Switzerland) and anti-CSPG. After incubation with biotinylated secondary antibodies and streptavidin–rhodamine complex, sections were analyzed in an epi-fluorescence microscope. To calculate the density of Cajal–Retzius (CR) cells, we counted the number of calretinin-positive neurons present in the marginal zone of the prospective parietal neocortex (two embryos and eight counts per group) (del Rio *et al.*, 1995).

For BrdU labeling, pregnant females were injected with a single BrdU dose at E10, E11, E13 or E15 (del Rio and Soriano, 1989). Offspring were killed at E18 and processed for the immunohistochemical detection of BrdU (del Rio and Soriano, 1989). The number of BrdU-positive neurons present in 250 μm thick vertical stripes covering the entire thickness of the parietal neocortex was counted (14–16 sections from 2–3 wild-type and mutant embryos per age). To analyze the radial distribution of positive neurons, the number of cells present in the marginal zone, subplate, intermediate zone, subventricular/ventricular zones, as well as in the lower, middle and upper tiers of the cortical plate, were counted. The radial distribution of labeled cells was analyzed as above.

Production of Recombinant Reelin

Reelin-conditioned media were prepared as described (Barallobre *et al.*, 2000; Keshvara *et al.*, 2001). The plasmid containing full-length Reelin cDNA or the empty vector were transfected into 293T cells using lipofectamine (Gibco-BRL). The next day culture medium was replaced by Optimem and cells were allowed to produce Reelin for 4 days. Conditioned media was collected, filtered through 0.22 μm porous membranes and concentrated ~60 times by using Millex GV filters (Millipore). Partially purified recombinant Reelin was a generous gift from Drs T. Curran and D. Benhayon (Memphis, TN). Reelin was purified by ammonium sulfate fractionation (Keshvara *et al.*, 2001, 2002).

Stimulation of Dissociated Neuronal Cultures

For dissociated cortical cultures, E15–E16 embryos were dissected out. Their brains were dissected in PBS containing 0.6% glucose and the neocortex and hippocampus were dissected out. After trypsin (Gibco-BRL) and DNase (Roche Diagnostics) treatments, tissue pieces were dissociated by gentle sweeping. Cells were then counted and seeded onto poly-D-lysine-coated dishes in Neurobasal medium containing 1% horse serum and B27 supplement (Gibco-BRL). In most experiments cells were seeded in six-well dishes at 1 million cells/well.

After 2 days *in vitro* (cells in culture), conditioned medium containing Reelin was diluted with fresh culture medium and added to the neuronal cultures for different periods of time. Partially purified Reelin was used at 2 ng/ml. Control experiments included incubation with conditioned media from control cells. In a few experiments, freshly dissociated cells were incubated with Reelin as described (Howell *et al.*, 1999a). After stimulation, cells were collected in 2× loading sample buffer for SDS–PAGE (0.15 M Tris, pH 6.5; 2-mercaptethanol 1 mM, SDS 1%, glycerol 10% and bromophenol blue 0.025%), boiled for 10 min and subjected to SDS–PAGE.

Protein Brain Extracts

The brains of *reeler* and *dab*1 mutant mice were collected at postnatal day (P) 0 and P5. The brains of *map1*B mutants were also collected at E18. Briefly, the hindbrains and forebrains of mutant mice and control littermates were homogenized in: HEPES 20 mM, pH 7, NaCl 150 mM, EGTA 5 mM, $MgCl_2$ 1 mM, glycerol 10%, aprotinin 1 mM, leupeptin 1 mM, PMSF 0.2 mM, NaF 0.1 M, sodium pyrophosphate 10 mM and sodium orthovanadate 0.2 mM. After centrifugation, supernatants were analyzed by Western blot.

Western Blot

Samples were loaded and run in 6% polyacrylamide gels at 150 V. After running, transfer to nitrocellulose membranes was performed in glycine 120 mM, Tris 125 mM and SDS 0.1% and methanol 20%. Transfer was performed at 300 mA for 2 h. Filters were then saturated in 3% BSA in TBS and incubated with the following antibodies: SMI 31 MAb (Sternberger Monoclonals) at 1/1000, 125 MAb (Gonzalez-Billault *et al.*, 2000), anti-tubulin (Roche Diagnostics) 1/1000, the NC19 antibody (Santa Cruz Biotechnology) 1/1000, anti-actin (Sigma) 1/1000, anti-GSK3 (Transduction Laboratories) 1/1000, and with anti-P-Tyr-GSK3 and anti-P-Ser-GSK3 (Bioscience International) 1/1000. Secondary antibodies were used at 1/2000 in TBS containing 3% powder milk. Labeling was visualized using ECL plus (Amersham). For immunodetection of Reelin we used the G10 MAb [1:10 000; generous gift of A. Goffinet (de Bergeyck *et al.*, 1998)]. For densitometric analyses the Quantity One (Bio-Rad) program was used. Densitometric analyses were normalized to total protein levels by the detection of tubulin, actin or total MAP1B (NC19 antibody). All the Western blot data represent a minimum of four separate experiments.

For the detection of total levels of mDAB1, samples from Reelin-treated cells were immunoprecipitated using the B3 antibody (Howell *et al.*, 1999a), followed by Western blot detection with the same antibody (1:1000). To determine phosphotyrosine mDAB1 levels, immunoprecipitates were subjected to Western blot with the 4G10 MAb (Upstate Biochemicals) as described (Howell *et al.*, 1999a).

Determination of GSK3 Activity

GSK3 assays were carried out as described (Sayas *et al.*, 1999). Cell extracts were prepared from hippocampal neurons at different times after addition of Reelin- or mock-transfected conditioned medium. Cells were collected with a scraper and homogenized in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM sodium ortohovanadate, 5 mM EDTA. The soluble fraction was immunoprecipitated with a GSK3 antibody (Transduction Laboratories). Samples of 10 μl were incubated in a buffer containing 25 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$ and a specific GSK3 substrate peptide [GS (Sayas *et al.*, 1999)] at a final concentration of 0.75 mg/ml, in the presence of [γ^{32}P]ATP. The reaction was stopped with 1% H_3PO_4. The difference between the kinase activities in the presence or absence of the GSK3 inhibitor LiCl (20 mM) was considered to reflect GSK3 kinase activity (Sayas *et al.*, 1999).

Pharmacological Inhibition of Protein Kinases

Neuronal cultures were prepared and trated with Reelin as above, except that dishes were coated with laminin (20 μg/ml) in some experiments. Cultures were supplemented with 10 mM lithium chloride and 20 mM myo-inositol to inhibit GSK3 activity (Sayas *et al.*, 2002) or with 250 nM roscovitine to inhibit CDK5 activity (Gonzalez-Billault *et al.*, 2001). In some experiments cultures were incubated with both inhibitors.

Expression of Reelin mRNA

Total RNA from *map1*B mutant and wild-type embryos were subjected to reverse transcription PCR reactions for the detection of Reelin and Actin mRNAs as described (Alvarez-Dolado *et al.*, 1999).

Results

Reelin Stimulates Mode I MAP1B Phosphorylation In Vitro *and* In Vivo

The response of developing neurons to Reelin is mediated through a complex signaling pathway comprising receptors, adaptor proteins and protein kinases (Walsh and Goffinet, 2000; Rice and Curran, 2001; Arnaud *et al.*, 2003; Bock *et al.*, 2003), which is believed to transduce the Reelin signal into cytoskeletal changes. MAP1B binds microtubules and actin microfilaments, contributing to their stabilization via a process that is believed to depend on type I MAP1B phosphorylation (Pedrotti and Islam 1996; Pigino *et al.*, 1997; Goold *et al.*, 1999). To examine whether Reelin can induce MAP1B mode I phosphorylation, we treated embryonic cortical neurons (E15–E16) with recombinant Reelin, a paradigm that has been shown to activate the Reelin signaling cascade (Howell *et al.*, 1999a; Keshvara *et al.*, 2001). Reelin-exposed neurons showed a dramatic increase in mode I MAP1B phosphorylation (e.g. ~15-fold at 1 h), which was maintained up to 6 h (Fig. 1*A,C*). The effects seemed to be specifically induced by Reelin, since 293T-conditioned media, derived from mock-transfected cells, failed to induce an increase in mode I MAP1B phosphorylation (Fig. 1*B*). Finally, incubation of cultured embryonic forebrain neurons with purified Reelin (2 ng/ml) yielded an increase in mode I MAP1B phosphorylation similar to that observed in the culture forebrain assay (Fig. 1*D,E*). In contrast, incubation with Reelin supernatant or with purified Reelin failed to induce type II MAP1B phosphorylation [detected with the 125 Mab; data not shown (see also Diaz-Nido *et al.*, 1988)]. Finally neither Reelin nor mock transfection altered the expression levels of MAP1B, as detected with the NC19 antibody (Fig. 1*A,B,D*).

The adaptor protein mDab1 is activated by the Reelin signaling pathway (Howell *et al.*, 1999a; Keshvara *et al.*, 2001). To confirm that the Reelin signaling cascade was activated in our experimental conditions, we have measured the levels of mDab1 phosphorylation by using anti-phosphotyrosine antibodies (Howell *et al.*, 1999a). Embryonic forebrain neurons treated with recombinant Reelin showed a significant increase in mDab1 phosphorylation (Fig. 1*F*, Reelin). Conversely, a similar treatment with mock transfected cells did not induce any significant change in mDab1 phosphorylation (Fig. 1*F*, mock). Changes were not due to differential protein expression, since total mDab1 levels were unaltered in all the above-mentioned conditions (Fig. 1*F*, lower panel).

If Reelin regulates mode I MAP1B phosphorylation *in vivo*, mice with genetic disruptions of the Reelin signaling pathway may have decreased levels of MAP1B phosphorylation. We first measured mode I MAP1B phosphorylation in protein extracts from Reelin-deficient *reeler* mice. The forebrains (P0) and hindbrains (P5) of *reeler* mice showed a 7-fold decrease in the levels of type I MAP1B phosphorylation, compared to wild-type littermates (Fig. 2*A,B* upper panels). Interestingly, heterozygous *reeler* mice displayed about a 2-fold reduction, indicating a dose-dependent effect. Subsequently, the forebrains and hindbrains of *m*D*ab*1 (–/–) newborn mice also showed decreased levels of mode I MAP1B phosphorylation (2-fold reduction, Fig. 2*A,B* lower panels). Together, these results indicate that Reelin stimulates mode I MAP1B phosphorylation in embryonic neurons *in vitro*, through a signaling pathway that involve mDab1 tyrosine phosphorylation. Thus, we conclude that MAP1B becomes phosphorylated *in vitro* and *in vivo* in response to Reelin in a signaling cascade which requires the adaptor protein mDab1. Interestingly, the amount of mode I MAP1B phosphorylation was not totally abolished in both *reeler* and *mDab1*$^{-/-}$ mutants, indicating that MAP1B phosphorylation is also modified by other signaling cascades. However, these data suggest that MAP1B phosphorylation can be indeed modified by the Reelin signaling pathway.

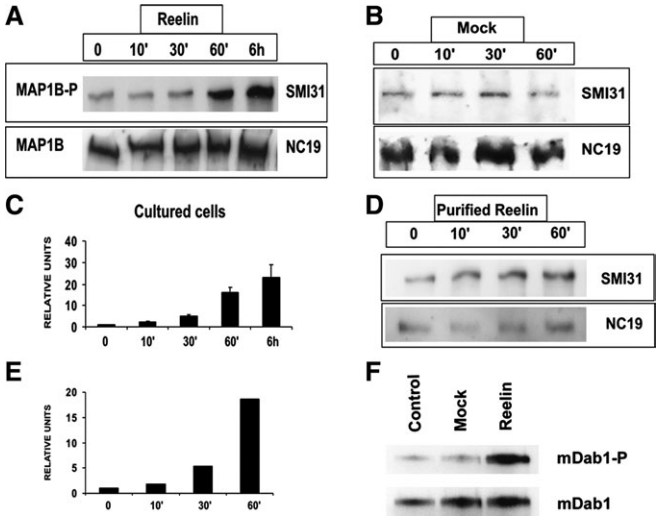

Figure 1. Reelin increases the levels of mode I MAP1B phosphorylation. (*A*) Western Blots illustrating induction of MAP1B phosphorylation by Reelin-conditioned media. Cortical neurons were cultured for 48 h and then treated with Reelin-conditioned medium for different times (0, 10, 30, 60 min and 6 h). Western blots of soluble cell extracts were incubated with antibodies against the mode I MAP1B phosphoepitope (SMI31), or total MAP1B (NC 19). α-Tubulin antibodies were used as loading controls. Reelin incubation results in a marked increase in mode I phosphorylation levels of MAP1B (SMI31). The phosphorylation levels of MAP1B protein in the SMI31 epitope were determined by densitometry and normalized respect to the total levels of MAP1B (NC19) in cultured cells (*C*). (*B*) MAP1B phosphorylation levels after treatment with mock-culture medium. Cortical neurons were cultured for 48 h and treated with conditioned medium from mock-transfected 293T cells for different times (10, 30, 60 min). The phosphorylation levels of MAP1B protein in the SMI31 epitope were determined by densitometry and normalized respect to the total levels of MAP1B (NC19) (data not shown). (*D*) MAP1B phosphorylation levels after treatment with partially purified Reelin. Western blots of soluble cell extracts from Reelin-treated or untreated neurons (0), were incubated with antibodies against MAP1B (SMI31 and NC19). The phosphorylation degree of MAP1B protein in the SMI31 epitope was determined by densitometry, normalized respect to the levels of total MAP1B (NC19), and graphically represented (*E*). (*F*) Reelin treatment induced mDab1 tyrosine phosphorylation. MDab1 protein was immunoprecipitated after exposure to Reelin or mock supernatant and further incubated with an anti-phosphotyrosine antibody. While mock-transfected cells did not induce a noticeable increase in mDab1 phosphorylation, Reelin-transfected cells increased phospho-tyrosine mDab1 levels.

Involvement of GSK3 and CDK5 Kinases in MAP1B Phosphorylation Triggered by Reelin

MAP1B is phosphorylated in two different ways by serine/threonine protein kinases: whereas GSK3 and CDK5 induces mode I phosphorylation, casein kinase II leads to mode II phosphorylation (Diaz-Nido *et al.*, 1988; Lucas *et al.*, 1998; Goold *et al.*, 1999; reviewed in Gonzalez-Billault *et al.*, 2004). A recent study reports that Reelin increases serine phosphorylation of GSK3 (Beffert *et al.*, 2002), a kinase that phosphorylates MAP1B in mode I. GSK3 activity is believed to be negatively regulated by serine phosphorylation (Beffert *et al.*, 2002). However, GSK3 is also phosphorylated at its Tyr 216, which is believed to increase GSK3 activity (Sayas *et al.*, 1999). Western

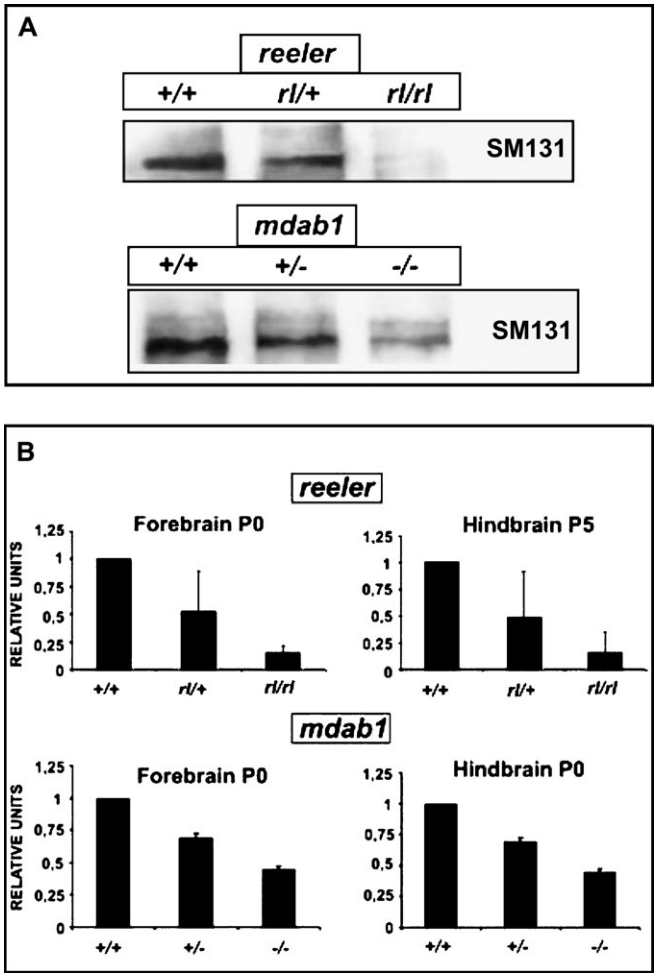

Figure 2. Reduced mode I MAP1B phosphorylation in Reeler and Dab1 −/− mutants. (*A*) Western blots illustrating mode I MAP1B phosphorylation levels in brain extracts from the forebrain (P0) of *reeler*, *rl*/+ and wild-type mice (upper panel), and from *mdab*1 (−/−), *mdab*1 (+/−) and wild-type littermates (lower panel). Western blots were incubated with the SMI31 antibody. (*B*) Histograms showing densitometric analysis of mode I MAP1B phosphorylation in the forebrains and hindbrains of *reeler* and *mdab1* mutant brains (mean ± SD; three cases each). The phosphorylation levels of MAP1B protein in the SMI31 epitope were determined as described above. The data was normalized respect to total MAP1B values (NC19), and graphically represented. The data from wild-type brains were referred as 1 relative unit. Note markedly decreased phosphorylation levels in homozygous and heterozygous mutant brains, compared to their wild-type littermates.

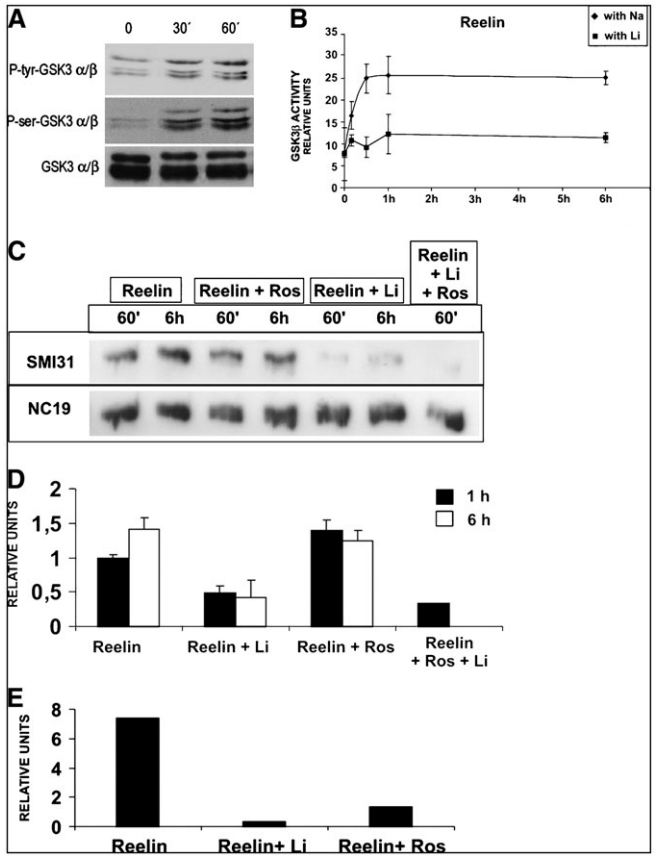

Figure 3. Contribution of GSK3 and CDK5 in the type I MAP1B phosphorylation induced by Reelin. (*A*) Western blots showing P-Tyr-GSK3 (upper panel), P-Ser-GSK3 (middle panel) and GSK3 total levels (lower panel) in cortical cultures treated with Reelin-conditioned media for 0–60 min, showing an increase of both GSK3 tyrosine and serine phosphorylation. (*B*) Plots illustrating the phosphorylation levels (mean ± SD) of a GSK3-specific peptide substrate in neuronal cultures after exposure to Reelin containing media for 0–6 h, in the presence or absence of LiCl, a GSK3 inhibitor. GSK3 kinase activity is considered to be the difference between the kinase activities in the presence (filled squares) or absence of LiCl (filled diamonds). Note that GSK3 activity increases shortly after Reelin exposition, consistent with an increase in tyrosine phosphorylation of the kinase; and the increased activity lasts for 6 h. (*C*) Western blot of soluble cell extracts from cortical neurons exposed to Reelin alone for 1 or 6 h, with Reelin and lithium chloride (Li), roscovitine (Ros), or both Li and Ros. Membranes were immunoreacted with the SMI31 MAb to visualize mode I MAP1B phosphorylation, or with the NC19 antibody. (*D*) Densitometric analysis of the Western blot shown in (*C*). The data were normalized with respect to the values corresponding to total MAP1B levels (NC19). (*E*) Densitometric analysis of mode I MAP1B phosphorylation levels as detected with the SMI31 MAb, normalized to total MAP1B levels. Embryonic cortical neurons were cultured on laminin-coated plates for 48 h, and then exposed for 1 h to Reelin alone, or to Reelin plus lithium (Li) or to Reelin plus roscovitine (Ros). Inhibition of either CDK5 (Ros) or GSK3 (Li) activities greatly reduces Reelin-induced MAP1B phosphorylation levels.

blots of cortical neurons incubated with Reelin supernatant showed increased levels of serine phosphorylation of GSK3 (Fig. 3*A*, middle panel), in agreement with the above study (Beffert *et al.*, 2002), but also increased tyrosine phosphorylation levels (Fig. 3*A*, upper panel). All the aforementioned effects were produced without affecting the total gsk3 protein levels (Fig. 3*A*, lower panel). Because serine and tyrosine phosphorylation levels may have opposite effects on GSK3 activity, we investigated the net effect of Reelin on GSK3 activity. To analyze GSK3 activity we performed an *in vitro* kinase activity assay, by incubating immunopurified enzyme from control and Reelin-treated neurons, and subsequently we measured its kinase activity on a specific peptide substrate. As shown in Figure 3*B*, there was a rapid increase in GSK3 enzymatic activity after exposure to Reelin, which lasted up to 6 h (Fig. 3*B*, filled diamonds). Interestingly, the increased activity of GSK3 after Reelin treatment was significantly inhibited by the addition of lithium chloride to the kinase assay (Fig. 3*B*, filled squares). Thus, we conclude that Reelin increases the phosphorylation of GSK3 at both serines and tyrosines, which in turn results in a marked increase in GSK3 enzymatic activity.

We have previously shown that a MAP1B phosphoepitope depending on GSK3 acitvation is increased on addition of Reelin (Fig. 1*A*). In order to confirm whether GSK3 plays a role in Reelin-induced MAP1B phosphorylation and to identify whether other kinases like CDK5 are responsible for Reelin-induced mode I phosphorylation of MAP1B, we used inhibitors of GSK3 and CDK5. Embryonic forebrain neurons treated with recombinant Reelin in the presence of LiCl (10 mM, a GSK3

inhibitor; Lucas *et al.* 1998) showed a marked decrease in the levels of mode I phosphorylation at 1 and 6 h (Fig. 3*C,D*). In contrast, incubation with the CDK5 inhibitor roscovitine (250 nM, Calbiochem) does not modify mode I MAP1B phosphorylation (Fig. 3*C,D*).

Since Reelin actions are believed to be mediated by CDK5, we investigated the contribution of CDK5. It is known that neurons cultured on laminin upregulate p35 expression, thus promoting CDK5 activity (Paglini *et al.*, 1998). We therefore performed similar experiments but with cultured forebrain neurons on laminin. Under these conditions, incubation with roscovitine inhibited mode I MAP1B phosphorylation induced by Reelin (Fig. 3*E*). Furthermore, inhibition of both GSK3 and CDK5 almost completely abolished mode I phosphorylation of MAP1B (Fig. 3*C,D*).

Altogether these results strongly suggest that MAP1B phosphorylation can be in part controlled by the Reelin signaling pathway, in a cascade that includes mDab1 tyrosine phosphorylation as well as GSK3 and CDK5 activation.

Map1*B-deficient Mice Show Migratory Deficits*

Because mode I of MAP1B phosphorylation could be induced after treatment with Reelin, we decided to investigate whether MAP1B function could be modified by Reelin. For this reason, in the first set of experiments we investigated if the *map1*B mutation affected the expression of Reelin. Sections from wild-type and mutant embryos were immunostained with calretinin antibodies to map the distribution of CR cells, the major source of Reelin at embryonic stages (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995; Alcantara *et al.*, 1998). No differences were observed in the distribution or the density of CR cells (18.7 ± 1.2 cells and 19.3 ± 0.9 cells per 250 μm in wild-type and mutant mice, respectively; two embryos and eight counts per group), indicating that MAP1B is not required either for the migration of CR cells or for their settlement in the marginal zone. To confirm that Reelin production was unaltered in mutant embryos, we measured Reelin expression by Western blot and RT-PCR. Again, the data confirmed that the levels of expression of both Reelin transcripts (Fig. 4*A*) and protein (Fig. 4*B*) were similar in wild-type and *map1*B mutant forebrains. Differences in the amount of Reelin mRNA between wild-type, heterozygous and homozygous groups were not significant when normalized against actin mRNA as internal control. At the protein level, we did not detect differences either in the 400 kDa protein or in the Reelin-related fragment of 180 kDa (Lambert de Rouvroit *et al.*, 1999). Again subtle differences appearing in the blot were not significant when normalized against tubulin as an internal control. Taking together, these results indicate that decreased Reelin levels cannot account for the migratory deficits detected in *map1*B-mutant embryos.

*Map1*B-deficient mice die soon after birth, therefore the phenotype of mutant embryos was analyzed at E18. In agreement with previous studies (Gonzalez-Billault *et al.*, 2000), Nissl-stained sections from *map1*B mutant embryos revealed structural abnormalities in certain laminated brain regions. Thus, although the neocortex of E18 mutant embryos contained the typical layers, i.e. the marginal zone, the cortical plate and the intermediate zone, the arrangement of postmitotic neurons in the cortical plate appeared disorganized. For instance, *map1*B-deficient neurons were arranged in a wave-like pattern in the lower cortical plate (corresponding to the prospective layers V and VI) (Fig. 5*A,B* arrows) — similar, but not indentical

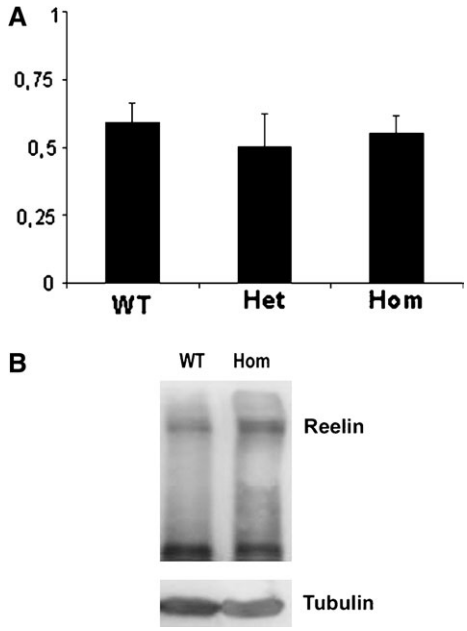

Figure 4. Reelin levels are unaltered in *map1b* mutants. (*A*) RT-PCR for *reelin* mRNA showing no significant variations amongst wild-type, heterozygous and homozygous animals. Samples were normalized against actin mRNA internal control. (*B*) Western blot of brain extracts derived from wild-type and homozygous animals showing no signicant variations in the levels of Reelin protein. Samples were normalized against tubulin as an internal control.

to that in *reeler* mice and mutants of components of the Reelin pathway (Fig. 5*C*). In other cortical areas, such as the hippocampus, homozygous mutant embryos showed a disorganized or bi-stratified pyramidal layer which was more evident in the CA3 region (Fig. 5*D,E*). Finally, the cerebella of *map1*B-deficient embryos were smaller and less foliated than wild-type littermates (Fig. 5*F,G*). Although Purkinje cells are not still arranged in a monolayer at E18, calbindin-immunostaining showed that mutant Purkinje cells appeared to be more widely distributed than wild-type Purkinje cells, also suggesting a lamination defect (Fig. 5*H,I*).

To examine migratory deficits in the neocortex of *map1*B-mutant mice, we pulse injected 5′-bromodeoxyuridine (BrdU) at E10–E15, and the positioning of labeled neurons was recorded in the parietal neocortex of E18 embryos. After E10 injections, labeled neurons in wild-type embryos were consistently found in the subplate and marginal zone, in agreement with earlier studies (Wood *et al.*, 1992; Price *et al.*, 1997) with a few cells located in the lower and upper cortical plate (Fig. 6*D*). Similar E10 injections in mutant embryos also labeled neurons in the marginal zone and subplate. However, mutant neurons were more widely distributed, and labeled neurons were also present in the intermediate zone, below the subplate (Fig. 6*D*). After pulses at E11, labeled neurons in wild-type embryos were mainly located in the subplate and in the deep cortical plate (Fig. 6*A,D*). In *map1*B-mutant embryos, labeled neurons were also present in these layers, but up to 23% of cells accumulated in the intermediate zone (Fig. 6*A,D*). To label cortical plate neurons we administered BrdU at E13 or E15. In wild-type embryos, E13 injections labeled many neurons in the deep and intermediate aspects of the cortical plate, corresponding to layers V and VI (Fig. 6*B,D*). E13-labeled neurons in homozygous mutant embryos were present in layers V and VI, but were also frequent in the intermediate zone (27%) and the

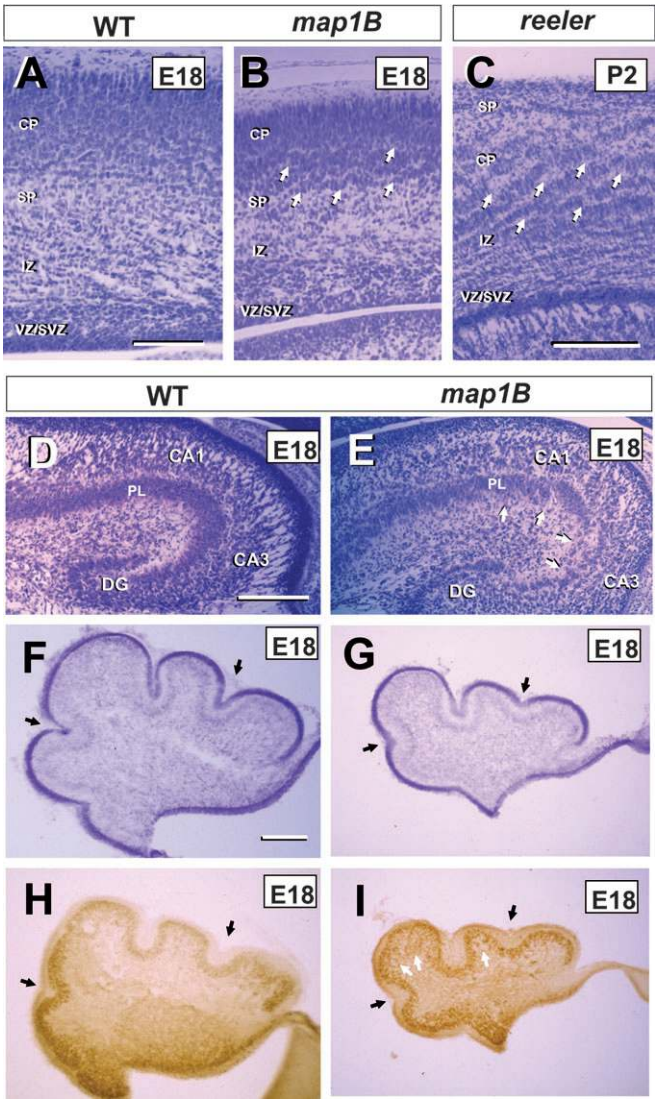

Figure 5. Migration abnormalities in *map1*B-deficient mice. (*A–C*) Laminar organization of the developing neocortex in wild-type embryos (*A*), *map1*B mutants (*B*) and in *reeler* mice (*C*). Nissl-staining. In *map1*B mutants (*B*), the subplate and the lower cortical plate show a disorganized, wavy distribution of neurons (white arrows), which resembles that in *reeler* mice (*C*). Scale bar, 200 μm. (*D*, *E*) Cytoarchitectonics of the embryonic hippocampus in wild-type (*C*) and *map1*B-deficient (*D*) embryos. Note the disorganized pyramidal layer (PL) (arrows in *E*) in the mutant hippocampus. Scale bar, 200 μm. (*F–I*) Cytoarchitectonics of the embryonic cerebellum in wild-type (*F*, *H*) and *map1*B mutant embryos (*G*, *I*). (*F*, *G*) are Nissl-stained sections. (*H*, *I*) are sections immunostained with calbindin antibodies. The mutant cerebellum displays smaller size and foliation defects (black arrows), compared to the wild-type cerebellum. Calbindin immunostaing shows that mutant Purkinje cells (*I*) appear to be more widespread distributed (white arrows) than wild-type Purkinje cells (*H*). Scale bar, 200 μm. Abbreviations: CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ, ventricular zone; SVZ, subventricular zone; CA1-CA3, hippocampal regions; DG, dentate gyrus.

subplate (21%) (Fig. 6*B*,*D*). Interestingly, many labeled neurons accumulated below the subplate in the mutant. Finally, E15 injections in wild-type embryos resulted in the accumulation of labeled neurons in the upper tier of the cortical plate (Fig. 6*C*,*D*). In contrast, labeled mutant neurons had a wider distribution in the three sublayers of the cortical plate (Fig. 6*C*,*D*).

To ascertain whether the preplate splits correctly in *map1*B mutant embryos, sections were stained with anti-calretinin or anti-chondroitin sulfate proteoglycan antibodies (CSPG; CS-56 antibody). Calretinin immunostaining showed that CR cells were correctly positioned in both wild-type and mutant mice, in the marginal zone (Fig. 7*C*,*F*). Similarly, CSPG immunostaining revealed that the subplate was positioned correctly in *map1*B mutant embryos (Fig. 7*B*,*E*). These results indicate that although the preplate appears to be split correctly into the marginal zone and the subplate in *map1*B-mutant mice, some cortical plate migrating neurons fail to settle appropriately into their correct cortical layer, suggesting abnormal migration of cortical neurons.

The present BrdU analyses in the neocortex, together with the lamination abnormalities in the pyramidal layer of the hippocampus and the cerebellum, indicate that *map1*B-deficient mice have migratory deficits that occur post-preplate splitting. Indeed, other post-preplate splitting mutants with neuronal migration defects have been described. Some of them have been related to the the Reelin signaling pathway such as *cdk*5 (–/–) and *p*35(–/–) mutant mice (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Gilmore *et al.*, 1998), while others, such as the Doublecortin mutant (Corbo *et al.*, 2002), have not.

Discussion

The present study shows migration abnormalities in the neocortex, hippocampus and more faintly in the cerebellum of *map1*B-deficient mice. No migration deficits have been reported in other strains of *map1*B-deficient mice which have been generated by distinct gene targeting approaches (Edelmann *et al.*, 1996; Takei *et al.*, 1997; Meixner *et al.*, 2000). In the first mutant line (Edelmann *et al.*, 1996), *map1*B-deficient animal die around E12, and in other mutant strains the lack of MAP1B gave rise to a mixture of phenotypes (Takei *et al.*, 1997, 2000; Gonzalez-Billault *et al.*, 2000; Meixner *et al.*, 2000). For this reason, interpretation of the phenotypes found in the latter *map1*B mutant lines had been somewhat controversial. A possible explanation for such discrepancies is the different genetic background among the various lines. It has been shown that genetic background can be responsible for dramatic changes in the phenotype of a mutated gene. For example, with the 129/Sv background, 100% of Pax-2-deficient mice display exencephaly in the midbrain/hindbrain region, whereas with a C57BL/6J background the frequency of this phenotype is reduced to 30% (Schwarz *et al.*, 1997). A similar effect has been described for engrailed-1 knockout mice (Bilovocky *et al.*, 2003). In agreement with this, null *map1*B mutants (Meixner *et al.*, 2000) and a hypomorph mutant expressing MAP1B alternative transcripts (Takei *et al.*, 2000) sharing the same genetic background, which is contributed by C57BL/6J and 129 strains, display incomplete penetration of the mutation that ultimately lead to lethality in 40–50% of the homozygous, and similar milder phenotypes, as compared with animals in the present study. In our mutant line, which contains contributions from NMRI and 129 strains, we verified a complete penetration of the mutation along with more severe phenotype (Gonzalez-Billault *et al.*, 2000), supporting a role for a contribution from the genetic background.

It has been proposed that the Reelin pathway transduces a signal that leads to cytoskeletal rearrangement and changes in cell motility (Walsh and Goffinet, 2000; Rice and Curran, 2001; Kawauchi *et al.*, 2003). The present study showing that MAP1B responds *in vitro* to extracellular Reelin by increasing mode I phosphorylation, and that Reelin-deficient mice have decreased levels of MAP1B mode I phosphorylation, supports the hypothesis that Reelin can trigger an increase in MAP1B phosphorylation,

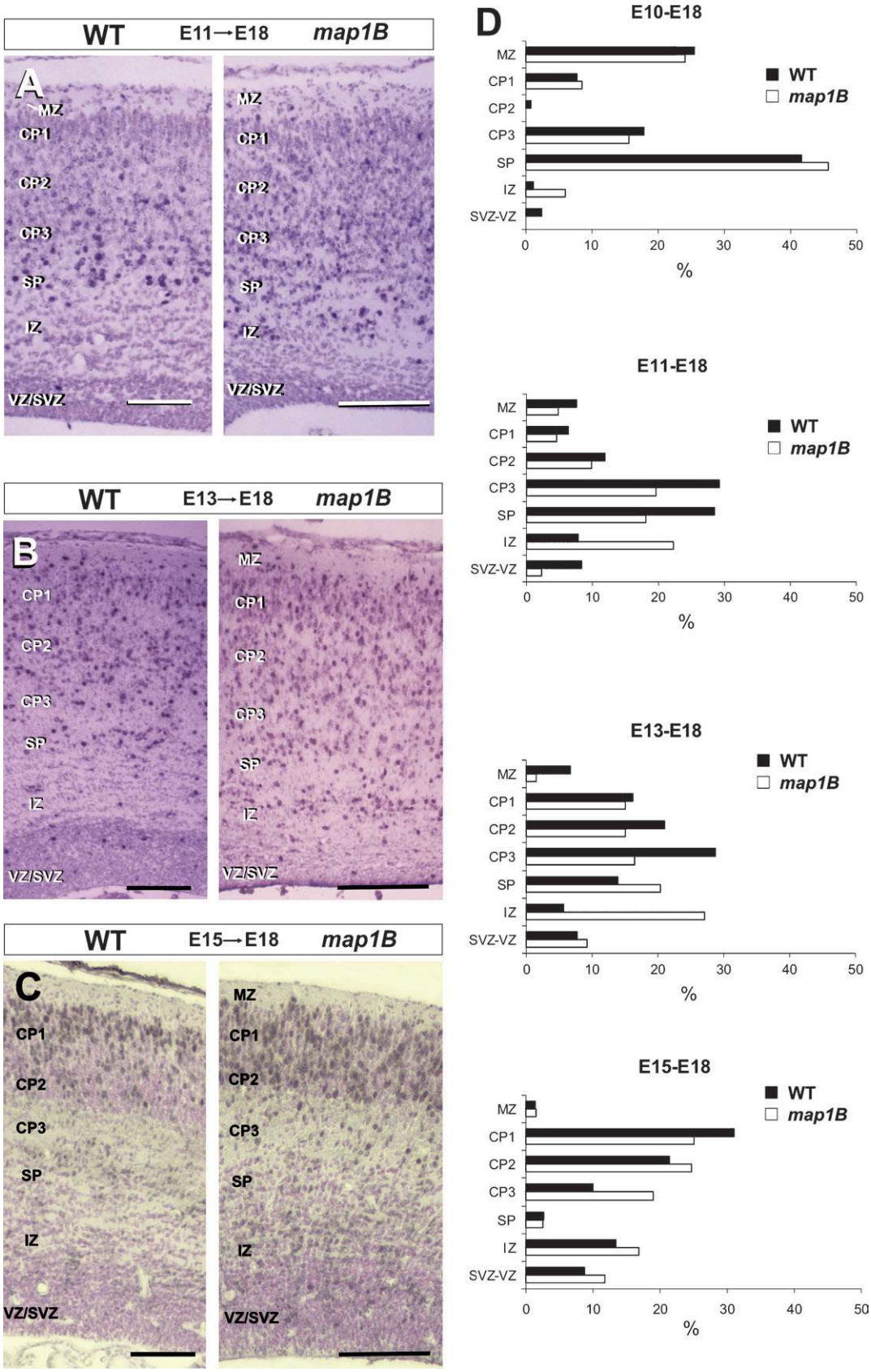

Figure 6. Distribution of BrdU-labeled neurons in the parietal neocortex. (*A–C*) Examples illustrating the distribution of neurons labeled by BrdU at E11 (*A*), E13 (*B*) or E15 (*C*), and analyzed at E18 in wild-type (left) and *map1*B mutant (right) embryos. Scale bars, 200 μm. (*D*) Radial distribution of BrdU-labeled neurons (in percentages) in the parietal neocortex of wild-type (black bars) and *map1*B mutant (white bars) embryos. Postmitotic neurons were labeled at E10, E11, E13 or E15, and were analyzed at E18. Cortical layers comprised the subventricular/ventricular zones (SVZ/VZ), intermediate zone (IZ), subplate (SP), marginal zone (MZ), and the lower, middle and upper tiers of the cortical plate (CP3, CP2, CP1).

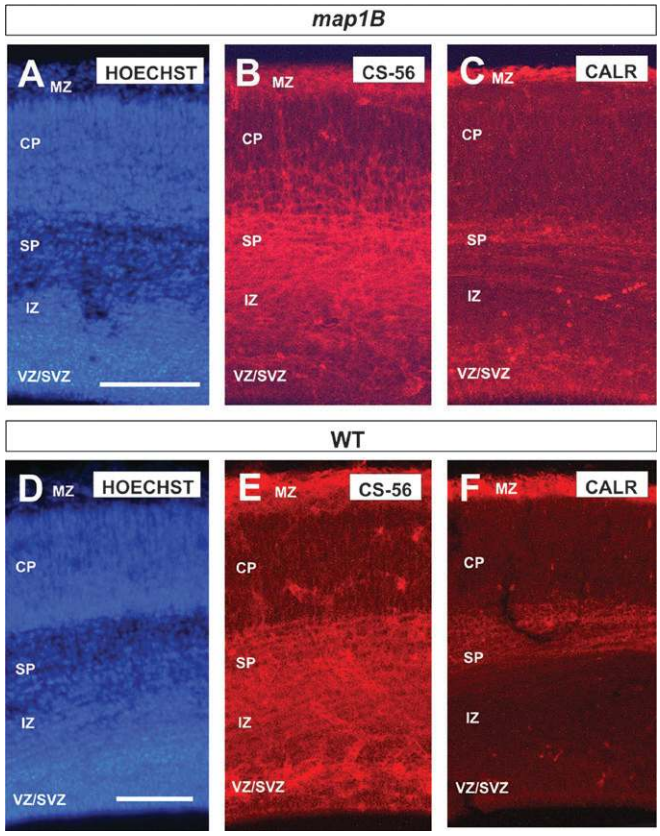

Figure 7. Distribution of subplate cells and Cajal–Retzius cells in the *map1*B mutant neocortex. (*A–F*) Comparison of the distribution of subplate cells (*B*, *E*: CS-56-immunoreactivity) and Cajal–Retzius cells (*C*, *F*: calretinin-immunoreactivity) in the *map1*B mutant and wild-type parietal neocortex at E18. No major differences were detected. (*A*) and (*D*) are sections counterstained with bisbenzimide, to illustrate the pattern of lamination. Abbreviations as in Figure 5.

both *in vivo* and *in vitro*. This view is reinforced by the finding that Reelin induces activation of GSK3, one of the enzymes responsible for mode I MAP1B phosphorylation (Sayas *et al.*, 1999). That MAP1B is modified *in vivo* by Reelin is further supported by the observation that disruption of the *mdab*1 gene, essential for transduction of the Reelin signal (Howell *et al.*, 1997, 1999a; Sheldon *et al.*, 1997), reduces mode I MAP1B phosphorylation. The fact that MAP1B is highly expressed in migrating neurons (Gonzalez-Billault *et al.*, 2000) is also consistent with this protein having a role in Reelin-dependent migration. Nevertheless, the fact that mode I MAP1B phosphorylation is not totally abolished in *Reeler* and *mDab1*$^{-/-}$ mutants suggests that other proteins can modulate MAP1B function. This seems to be case for Netrin-1, an extracellular cue participating in neuronal migration and axonal guidance (Tessier-Lavigne and Goodman, 1996). Netrin-1 is also able to induce MAP1B phosphorylation, and several neuronal tracts responding to Netrin-1 effects are mispositioned in MAP1B mutants (del Rio *et al.*, 2004). Moreover, neuronal migration of pontine nuclei neurons, which is also dependent on Netrin-1 function, is dramatically impaired in MAP1B mutants (del Rio *et al.*, 2004).

The migratory deficits observed in *map1*B mutant embryos in the neocortex, hippocampus and cerebellum are less dramatic than those in *reeler* mutant mice. Thus, cortical plate neurons are diffusely positioned in the cortical plate and intermediate zone, the hippocampal pyramidal layer is disrupted, and the cerebellum has foliation abnormalities (Rice and Curran, 2001). However, unlike *reeler*, the preplate splits correctly into the marginal zone and subplate in *map1*B-deficient mice, although mutant subplate neurons are ectopically positioned in the intermediate zone. Also the cerebellar phenotype for Purkinje cells is less dramatic than in *reeler*. Similar variation in the severity of migration deficits has been reported in mutations of several genes which are believed to act downstream of the Reelin pathway. For instance, mice targeted for the *cdk5* or *p35* genes (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Gilmore *et al.*, 1998; Kwon and Tsai, 1998; Ko *et al.*, 2001) also show a correct splitting of the preplate, but with migration defects in later-generated neurons that should migrate to the cortical and hippocampal plates. Moreover, *p35* mutations lead to mild effects in the positioning of neocortical and hippocampal neurons (Chae *et al.*, 1997), which are severed in double *p35/p39* mutants (Ko *et al.*, 2001). In contrast, mutations in the *dab1* gene or double mutations of the Reelin receptors (ApoER2 and LDLR) show migration deficits almost identical to those in *reeler* (Howell *et al.*, 1997; Sheldon *et al.*, 1997). These data support the hypothesis that mutation in genes acting progressively more downstream in the Reelin cascade results in progressively less severe phenotypic alterations, which is consistent with the present findings in *map1*B mutants. It is also possible that other cytoskeletal-associated proteins, with functional redundancy to MAP1B, participate in the Reelin signaling pathway. This notion is also strengthened by recent findings that LIS1 could be either an obligatory component of the Reelin pathway that functions downstream of mDab1 or could be a component of just one of several branches that constitute the intracellular response to Reelin (Assadi *et al.*, 2003). LIS1 is another microtubule-associated protein that when mutated produces neuronal migration defects that occur after preplate splitting. Analogously, LIS1 mutants display migration abnormalities that are not identical to the *reeler* mutant (Hirotsune *et al.*, 1998; Cahana *et al.*, 2001).

Proline-directed protein kinases such as GSK3 and CDK5 have been implicated in mode I MAP1B phosphorylation (Pigino *et al.*, 1997; Lucas *et al.*, 1998; Garcia-Perez *et al.*, 1998; Goold *et al.*, 1999). Our pharmacological experiments with lithium — which blocks GSK3 activity and MAP1B phosphorylation — indicates an essential role of this kinase in Reelin-induced mode I MAP1B phosphorylation. Notably, it has recently been reported that the effects of lithium, specifically upon MAP1B phosphorylation and axonal elongation, are indistinguishable from those of the specific GSK3 inhibitor SB-216763 (Owen and Gordon-Weeks, 2003). Finally, the implication of GSK3 in mode I MAP1B phosphorylation after Reelin treatment is also supported by the increase in GSK3β kinase activity. The analysis of CDK5 contribution was more complex, since in our experiments Reelin-induced mode I phosphorylation was not blocked by roscovitine. This could be due to the fact that neurons were initially cultured on poly-L-lysine, a substrate that does not favor CDK5 activity (Paglini *et al.*, 1998). When embryonic neurons were cultured on laminin, which increases CDK5 activity, roscovitine decreased mode I MAP1B phosphorylation. Because embryonic neurons *in vivo* develop in a rich extracellular 'milieu', composed of many extracellular proteins, and since CDK5 may be essential for the Reelin signaling (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Rice and Curran, 2001; Smith and Tsai, 2002), we propose that both GSK3 and CDK5 cooperate *in vivo* in the Reelin-induced mode I phosphorylation of MAP1B. In fact, pharmacological blockade of both GSK3 and CDK5 almost

completely abolished type I MAP1B phosphorylation. This is consistent with a synergistic effect for those kinases. In fact, several reports have indicated that many GSK3 substrates must be previously phosphorylated in the –4 position by other protein kinases, including CDK5, to be then modified by GSK3 (Cohen and Frame, 2001). Indeed, the MAP1B phospho-epitope recognized by the SMI31 MAb contains different serines at a +4 position that could be modified by *cdk5*. Thus, we propose that MAP1B can be phosphorylated by CDK5 to act as a primer to favor GSK3 modification.

It is believed that CDK5 is a downstream effector in the Reelin pathway, although there is no evidence of activation of CDK5 by Reelin (Zukerberg *et al.*, 2000; Smith and Tsai, 2002). In addition, mDab1 is phosphorylated by CDK5 in a Reelin-independent manner (Keshvara *et al.*, 2001). A recent study reports that Reelin regulates serine phosphorylation of GSK3, suggesting an inhibition of enzymatic activity (Beffert *et al.*, 2002). However, GSK3 has two isoforms, α and β, and its activity is downregulated by phosphorylation of serines and upregulated by phosphorylation on tyrosines (Cohen and Frame, 2001; Grimes and Jope, 2001). In contrast, our study shows that Reelin increases phosphorylation of both serines and tyrosines of GSK3, resulting in a marked increase in GSK3 enzymatic activity. This observation is also consistent with that indicating that phosphorylation of MAP1B, a GSK3 substrate, increases upon exposure to Reelin (Fig. 8). Several pathways have been proposed to inhibit GSK3 activity, including the insulin/IGF-I pathway through activation of the PI3K-Akt/PKB pathway (Cohen and Frame, 2001; Grimes and Jope, 2001) and the Wnt/Wingless signaling cascade (Welsh and Proud, 1993; Cook *et al.*, 1996). In contrast, only a few studies report activation of GSK3 by FGF2 or LPA (Sayas *et al.*, 1999, 2002; Hashimoto *et al.*, 2002) by mechanisms that remain largely unknown. We have shown here that the Reelin signaling pathway also activates GSK3 (Fig. 8).

The present analyses *in vivo* and *in vitro* indicate that MAP1B can be modified by the Reelin signaling pathway. It is thought that both microtubules and actin filaments, which are concentrated at the leading process of migrating neurons, are essential for neuronal migration (Rivas and Hatten, 1995). MAP1B has been shown to control the dynamic properties of microtubules (Pedrotti and Islam, 1996; Togel *et al.*, 1998). For instance, it has been suggested that cultured *map1*B-deficient neurons contain fewer microtubules than control cells (Gonzalez-Billault *et al.*, 2001). Moreover, in the absence of MAP1B, the number of dynamic microtubules in the distal part of the axons is much lower, whereas the number and distribution of stable microtubules increase (Gonzalez-Billault *et al.*, 2001).

We also show that mode I MAP1B phosphorylation is activated by Reelin. Mode I phosphorylated MAP1B is upregulated during development and is present in a gradient-dependent manner in axons and neurites, being enriched in the distal part (Riederer *et al.*, 1990; Ulloa *et al*, 1993, 1994; Mack *et al.*, 2000). At the cellular level, inactivation of GSK3 or CDK5 kinases induces a neural phenotype *in vitro* that resembles that of *map1*B-deficient neurons (Pigino *et al.*, 1997; Lucas *et al.*, 1998; Paglini *et al*, 1998; Goold *et al.*, 1999; Gonzalez-Billault *et al.*, 2002). Moreover, ablation of mode I MAP1B phosphorylation by micro-CALI experiments affects growth cone tuning in cultured neurons (Mack *et al.*, 2000). Finally, it has been reported that overexpression of Wnt7b, a signal that inactivates GSK3 inducing a decrease of MAP1B phosphorylation in cortical explants, leads to abnormal positioning of cortical neurons (Viti *et al.*, 2003). All these data have led to the suggestion that most functions of MAP1B are regulated by mode-I phosphorylation, which is likely to increase microtubule and actin instability and cross-talk (Goold *et al.*, 1999; Mack *et al.*, 2000).

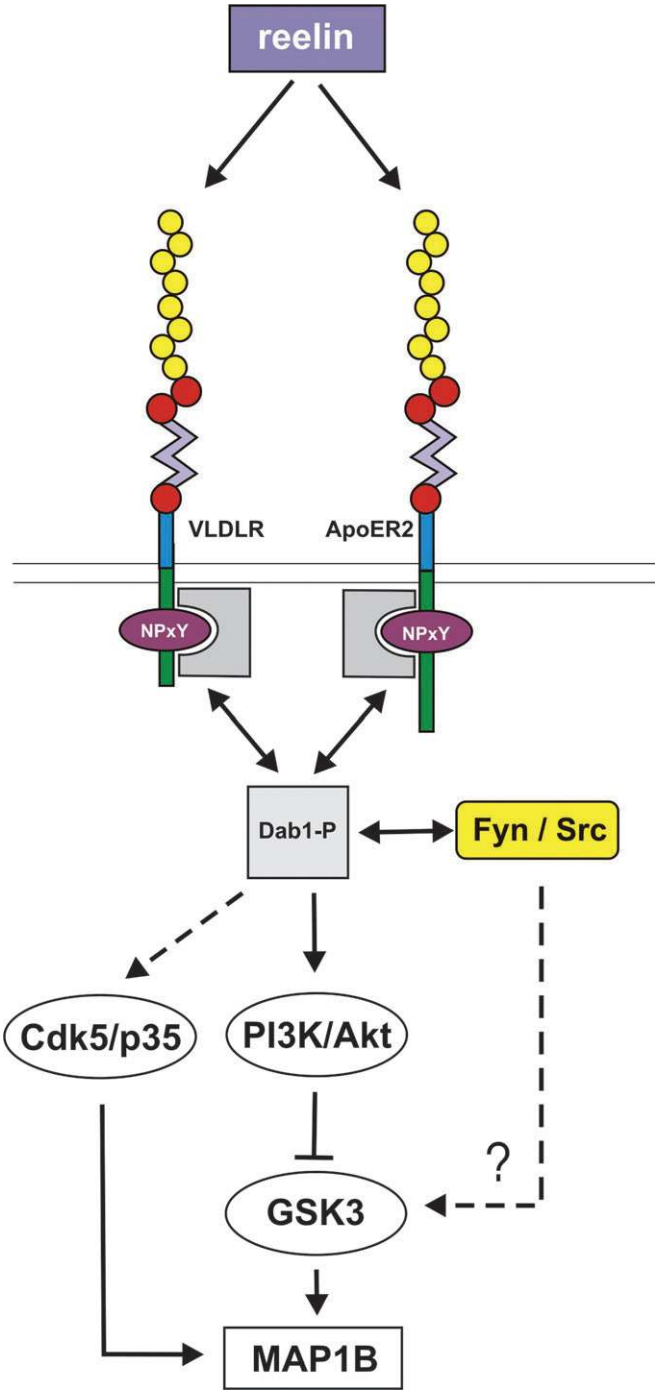

Figure 8. Schematic diagram showing the Reelin signaling pathway. The extracellular protein Reelin binds to the VLDLR and ApoER2 receptors. The adaptor protein Dab1 associates with the NPxY motifs in the cytoplasmic domains of these receptors. Reelin binding to their receptors activates Src/Fyn tyrosine kinases, resulting in phosphorylation of Dab1. Furthermore, the Reelin signaling pathway activates a complex array of intracellular cascades including PI3K/Akt, Cdk5 and probably other unknown kinases. These intracellular kinases control GSK3 activity, which, together with CDK5, phosphorylate MAP1B. Mutations in the *reelin* and *dab-1* genes elicit a similar decrease in mode I-MAP1B phosphorylation.

In conclusion, we propose that MAP1B function can be modified by the Reelin signaling pathway. Absence of MAP1B can to some extent mimic alterations in the Reelin pathway, producing abnormal migration of neurons. Nevertheless, MAP1B function could also be altered by other extracellular cues, acting either independently or in paralel with Reelin. Finally, the increase in MAP1B phosphorylation is dependent on GSK3 and CDK5 protein kinases.

Notes

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References

Alcantara S, Ruiz M, D'Arcangelo G, Ezan F, de Lecea L, Curran T, Sotelo C, Soriano E (1998) Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. J Neurosci 18:7779–7799.

Alvarez-Dolado M, Ruiz M, Del Rio JA, Alcantara S, Burgaya F, Sheldon M, Nakajima K, Bernal J, Howell BW, Curran T, Soriano E, Munoz A (1999) Thyroid hormone regulates reelin and dab1 expression during brain development. J Neurosci 19:6979–6993.

Arnaud L, Ballif BA, Forster E, Cooper JA (2003) Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development. Curr Biol 13:9–17.

Assadi AH, Zhang G, Beffert U, McNeil RS, Renfro AL, Niu S, Quattrocchi CC, Antalffy BA, Sheldon M, Armstrong DD, Wynshaw-Boris A, Herz J, D'Arcangelo G, Clark GD (2003) Interaction of reelin signaling and Lis1 in brain development. Nat Genet 35:270–276.

Avila J, Dominguez J, Diaz-Nido J (1994a) Regulation of microtubule dynamics by microtubule-associated protein expression and phosphorylation during neuronal development. Int J Dev Biol 38: 13–25.

Avila J, Ulloa L, Diez-Guerra J, Diaz-Nido J (1994b) Role of phosphorylated MAPlB in neuritogenesis. Cell Biol Int 18:309–314.

Barallobre MJ, Del Rio JA, Alcantara S, Borrell V, Aguado F, Ruiz M, Carmona MA, Martin M, Fabre M, Yuste R, Tessier-Lavigne M, Soriano E (2000) Aberrant development of hippocampal circuits and altered neural activity in netrin 1-deficient mice. Development 127: 4797–4810.

Beffert U, Morfini G, Bock HH, Reyna H, Brady ST, Herz J (2002) Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta. J Biol Chem 277:49958–49964.

Bilovocky NA, Romito-DiGiacomo RR, Murcia CL, Maricich SM, Herrup K (2003) Factors in the genetic background suppress the engrailed-1 cerebellar phenotype. J Neurosci 23:5105–5112.

Bock HH, Jossin Y, Liu P, Forster E, May P, Goffinet AM, Herz J (2003) Phosphatidylinositol 3-kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination. J Biol Chem 278:38772–38779.

Cahana A, Escamez T, Nowakowski RS, Hayes NL, Giacobini M, von Holst A, Shmueli O, Sapir T, McConnell SK, Wurst W, Martinez S, Reiner O (2001) Targeted mutagenesis of Lis1 disrupts cortical development and LIS1 homodimerization. Proc Natl Acad Sci USA 98:6429–6434.

Chae T, Kwon YT, Bronson R, Dikkes P, Li E, Tsai LH (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron 18:29–42.

Chowdhury K, Bonaldo P, Torres M, Stoykova A, Gruss P (1997) Evidence for the stochastic integration of gene trap vectors into the mouse germline. Nucleic Acids Res 25:1531–1536.

Cohen P, Frame S (2001) The renaissance of GSK3. Nat Rev Mol Cell Biol 2:769–776.

Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR, Dale TC (1996) Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. Embo J 15:4526–4536.

Corbo JC, Deuel TA, Long JM, LaPorte P, Tsai E, Wynshaw-Boris A, Walsh CA (2002) Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. J Neurosci 22:7548–7557.

D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T (1999) Reelin is a ligand for lipoprotein receptors. Neuron 24:471–479.

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374:719–723.

de Bergeyck V, Naerhuyzen B, Goffinet AM, Lambert de Rouvroit C (1998) A panel of monoclonal antibodies against reelin, the extracellular matrix protein defective in reeler mutant mice. J Neurosci Methods 82:17–24.

del Rio JA, Soriano E (1989) Immunocytochemical detection of 5′-bromodeoxyuridine incorporation in the central nervous system of the mouse. Brain Res Dev Brain Res 49:311–317.

del Rio JA, Martinez A, Fonseca M, Auladell C, Soriano E (1995) Glutamate-like immunoreactivity and fate of Cajal–Retzius cells in the murine cortex as identified with calretinin antibody. Cereb Cortex 5:13–21.

del Rio JA, Gonzalez-Billault C, Ureña JM, Jimenez EM, Barallobre MJ, Pascual M, Pujadas Ll, Simo S, La Torre A, Wandosell F, Avila J, Soriano E (2004) MAP1B is required for netrin signaling in neuronal migration and axonal guidance. Curr Biol 14:840–850.

Diaz-Nido J, Serrano L, Mendez E, Avila J (1988) A casein kinase II-related activity is involved in phosphorylation of microtubule-associated protein MAP-1B during neuroblastoma cell differentiation. J Cell Biol 106:2057–2065.

Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, Kreidberg JA, Anton ES (2000) Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron 27:33–44.

Edelmann W, Zervas M, Costello P, Roback L, Fischer I, Hammarback JA, Cowan N, Davies P, Wainer B, Kucherlapati R (1996) Neuronal abnormalities in microtubule-associated protein 1B mutant mice. Proc Natl Acad Sci USA 93:1270–1275.

Feng Y, Walsh CA (2001) Protein–protein interactions, cytoskeletal regulation and neuronal migration. Nat Rev Neurosci 2:408–416.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247–256.

Garcia-Perez J, Avila J, Diaz-Nido J (1998) Implication of cyclin-dependent kinases and glycogen synthase kinase 3 in the phosphorylation of microtubule-associated protein 1B in developing neuronal cells. J Neurosci Res 52:445–452.

Gilmore EC, Ohshima T, Goffinet AM, Kulkarni AB, Herrup K (1998) Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J Neurosci 18:6370–6377.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257–271.

Gonzalez-Billault C, Demandt E, Wandosell F, Torres M, Bonaldo P, Stoykova A, Chowdhury K, Gruss P, Avila J, Sanchez MP (2000)

Perinatal lethality of microtubule-associated protein 1B-deficient mice expressing alternative isoforms of the protein at low levels. Mol Cell Neurosci 16:408–421.
Gonzalez-Billault C, Avila J, Caceres A (2001) Evidence for the role of MAP1B in axon formation. Mol Biol Cell 12:2087–2098.
Gonzalez-Billault C, Owen R, Gordon-Weeks PR, Avila J (2002) Microtubule-associated protein 1B is involved in the initial stages of axonogenesis in peripheral nervous system cultured neurons. Brain Res 943:56–67.
Gonzalez-Billault C, Jimenez-Mateos EM, Caceres A, Diaz-Nido J, Wandosell F, Avila J (2004) Microtubule-associated protein 1B function during normal development, regeneration and pathological conditions in the nervous system. J Neurobiol 58:48–59.
Goold RG, Owen R, Gordon-Weeks PR (1999) Glycogen synthase kinase 3beta phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. J Cell Sci 112:3373–3384.
Grimes CA, Jope RS (2001) CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. J Neurochem 78:1219–1232.
Hashimoto T, Nakano Y, Yamashita M, Fang YI, Ohata H, Momose K (2002) Role of Rho-associated protein kinase and histamine in lysophosphatidic acid-induced airway hyperresponsiveness in guinea pigs. Jpn J Pharmacol 88:256–261.
Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J (1999) Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron 24:481–489.
Hirotsune S, Takahara T, Sasaki N, Hirose K, Yoshiki A, Ohashi T, Kusakabe M, Murakami Y, Muramatsu M, Watanabe S, *et al.* (1995) The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. Nat Genet 10:77–83.
Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, Clark GD, Ledbetter DH, McBain CJ, Wynshaw-Boris A (1998) Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. Nat Genet 19:333–339.
Howell BW, Hawkes R, Soriano P, Cooper JA (1997) Neuronal position in the developing brain is regulated by mouse disabled-1. Nature 389:733–737.
Howell BW, Herrick TM, Cooper JA (1999a) Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. Genes Dev 13:643–648.
Howell BW, Lanier LM, Frank R, Gertler FB, Cooper JA (1999b) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. Mol Cell Biol 19:5179–5188.
Hummel T, Krukkert K, Roos J, Davis G, Klambt C (2000) *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron 26:357–370.
Ishiguro K, Kobayashi S, Omori A, Takamatsu M, Yonekura S, Anzai K, Imahori K, Uchida T (1994) Identification of the 23 kDa subunit of tau protein kinase II as a putative activator of cdk5 in bovine brain. FEBS Lett 342:203–208.
Kawauchi T, Chihama K, Nabeshima Y, Hoshino M (2003) The *in vivo* roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. Embo J 22:4190–4201.
Keshvara L, Benhayon D, Magdaleno S, Curran T (2001) Identification of reelin-induced sites of tyrosyl phosphorylation on disabled 1. J Biol Chem 276:16008–16014.
Keshvara L, Magdaleno S, Benhayon D, Curran T (2002) Cyclin-dependent kinase 5 phosphorylates disabled 1 independently of Reelin signaling. J Neurosci 22:4869–4877.
Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E, Tsai LH (2001) p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J Neurosci 21:6758–6771.
Kwon YT, Tsai LH (1998) A novel disruption of cortical development in p35(−/−) mice distinct from reeler. J Comp Neurol 395:510–522.
Lambert de Rouvroit C, de Bergeyck V, Cortvrindt C, Bar I, Eeckhout Y, Goffinet AM (1999) Reelin, the extracellular matrix protein deficient in reeler mutant mice, is processed by a metalloproteinase. Exp Neurol 156:214–217.
Lucas FR, Goold RG, Gordon-Weeks PR, Salinas PC (1998) Inhibition of GSK-3beta leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. J Cell Sci 111:1351–1361.
Mack TG, Koester MP, Pollerberg GE (2000) The microtubule-associated protein MAP1B is involved in local stabilization of turning growth cones. Mol Cell Neurosci 15:51–65.
Meixner A, Haverkamp S, Wassle H, Fuhrer S, Thalhammer J, Kropf N, Bittner RE, Lassmann H, Wiche G, Propst F (2000) MAP1B is required for axon guidance and is involved in the development of the central and peripheral nervous system. J Cell Biol 151:1169–1178.
Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K (1995) The reeler gene-associated antigen on Cajal–Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14:899–912.
Ohshima T, Ward JM, Huh CG, Longenecker G, Veeranna, Pant HC, Brady RO, Martin LJ, Kulkarni AB (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc Natl Acad Sci USA 93:11173–11178.
Owen R, Gordon-Weeks PR (2003) Inhibition of glycogen synthase kinase 3beta in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. Mol Cell Neurosci 23:626–637.
Paglini G, Pigino G, Kunda P, Morfini G, Maccioni R, Quiroga S, Ferreira A, Caceres A (1998) Evidence for the participation of the neuron-specific CDK5 activator P35 during laminin-enhanced axonal growth. J Neurosci 18:9858–9869.
Pedrotti B, Islam K (1996) Dephosphorylated but not phosphorylated microtubule associated protein MAP1B binds to microfilaments. FEBS Lett 388:131–133.
Pedrotti B, Ulloa L, Avila J, Islam K (1996) Characterization of microtubule-associated protein MAP1B: phosphorylation state, light chains, and binding to microtubules. Biochemistry 35:3016–3023.
Pigino G, Paglini G, Ulloa L, Avila J, Caceres A (1997) Analysis of the expression, distribution and function of cyclin dependent kinase 5 (cdk5) in developing cerebellar macroneurons. J Cell Sci 110:257–270.
Price DJ, Aslam S, Tasker L, Gillies K (1997) Fates of the earliest generated cells in the developing murine neocortex. J Comp Neurol 377:414–422.
Rice DS, Curran T (2001) Role of the reelin signaling pathway in central nervous system development. Annu Rev Neurosci 24:1005–1039.
Riederer BM, Guadano-Ferraz A, Innocenti GM (1990) Difference in distribution of microtubule-associated proteins 5a and 5b during the development of cerebral cortex and corpus callosum in cats: dependence on phosphorylation. Brain Res Dev Brain Res 56:235–243.
Rivas RJ, Hatten ME (1995) Motility and cytoskeletal organization of migrating cerebellar granule neurons. J Neurosci 15:981–989.
Roos J, Hummel T, Ng N, Klambt C, Davis GW (2000) *Drosophila* Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. Neuron 26:371–382.
Sayas CL, Moreno-Flores MT, Avila J, Wandosell F (1999) The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like Tau phosphorylation. J Biol Chem 274:37046–37052.
Sayas CL, Avila J, Wandosell F (2002) Glycogen synthase kinase-3 is activated in neuronal cells by Galpha12 and Galpha13 by Rho-independent and Rho-dependent mechanisms. J Neurosci 22:6863–6875.
Schwarz M, Alvarez-Bolado G, Urbanek P, Busslinger M, Gruss P (1997) Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. Proc Natl Acad Sci USA 94:14518–14523.
Senzaki K, Ogawa M, Yagi T (1999) Proteins of the CNR family are multiple receptors for Reelin. Cell 99:635–647.
Sheldon M, Rice DS, D'Arcangelo G, Yoneshima H, Nakajima K, Mikoshiba K, Howell BW, Cooper JA, Goldowitz D, Curran T (1997) Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature 389:730–733.

Smith DS, Tsai LH (2002) Cdk5 behind the wheel: a role in trafficking and transport? Trends Cell Biol 12:28–36.

Takei Y, Kondo S, Harada A, Inomata S, Noda T, Hirokawa N (1997) Delayed development of nervous system in mice homozygous for disrupted microtubule-associated protein 1B (MAP1B) gene. J Cell Biol 137:1615–1626.

Takei Y, Teng J, Harada A, Hirokawa N (2000) Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. J Cell Biol 150:989–1000.

Teng J, Takei Y, Harada A, Nakata T, Chen J, Hirokawa N (2001) Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. J Cell Biol 155:65–76.

Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274:1123–1133.

Togel M, Wiche G, Propst F (1998) Novel features of the light chain of microtubule-associated protein MAP1B: microtubule stabilization, self interaction, actin filament binding, and regulation by the heavy chain. J Cell Biol 143:695–707.

Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA, Herz J (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97:689–701.

Ulloa L, Diaz-Nido J, Avila J (1993) Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells. Embo J 12:1633–1640.

Ulloa L, Diez-Guerra FJ, Avila J, Diaz-Nido J (1994) Localization of differentially phosphorylated isoforms of microtubule- associated protein 1B in cultured rat hippocampal neurons. Neuroscience 61:211–223.

Viti J, Gulacsi A, Lillien L (2003) Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. J Neurosci 23:5919–5927.

Walsh CA, Goffinet AM (2000) Potential mechanisms of mutations that affect neuronal migration in man and mouse. Curr Opin Genet Dev 10:270–274.

Welsh GI, Proud CG (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. Biochem J 294:625–629.

Wood JG, Martin S, Price DJ (1992) Evidence that the earliest generated cells of the murine cerebral cortex form a transient population in the subplate and marginal zone. Brain Res Dev Brain Res 66:137–140.

Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA, Tsai LH (2000) Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron 26:633–646.