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Microtubule-Associated Protein 1B Interaction with Tubulin Tyrosine Ligase Contributes to the Control of Microtubule Tyrosination

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Neuronal migration · Axonogenesis · Microtubule dynamics · Microtubule-associated proteins

Abstract
Microtubule-associated protein 1B (MAP1B) is the first microtubule-associated protein to be expressed during nervous system development. MAP1B belongs to a large family of proteins that contribute to the stabilization and/or enhancement of microtubule polymerization. These functions are related to the control of the dynamic properties of microtubules. The C-terminal domain of the neuronal α-tubulin isotype is characterized by the presence of an acidic polypeptide, with the last amino acid being tyrosine. This tyrosine residue may be enzymatically removed from the protein by an unknown carboxypeptidase activity. Subsequently, the tyrosine residue is again incorporated into this tubulin by another enzyme, tubulin tyrosine ligase, to yield tyrosinated tubulin. Because neurons lacking MAP1B have a reduced proportion of tyrosinated microtubules, we analyzed the possible interaction between MAP1B and tubulin tyrosine ligase. Our results show that these proteins indeed interact and that the interaction is not affected by MAP1B phosphorylation. Additionally, neurons lacking MAP1B, when exposed to drugs that reversibly depolymerize microtubules, do not fully recover tyrosinated microtubules upon drug removal. These results suggest that MAP1B regulates tyrosination of α-tubulin in neuronal microtubules. This regulation may be important for general processes involved in nervous system development such as axonal guidance and neuronal migration.

E.U. and E.M.J.-M. equally contributed to this work.

Introduction

Neurons are polarized cells that contain a single long axon and many dendrites. Polarization of spherical neuroblasts occurs when these cells project distinct neurites, one of them being the future axon [Dotti et al., 1988]. It has been suggested that microtubule assembly and stabilization play an important role in axonogenesis [Mitchi-

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son and Kirschner, 1988]; among the microtubule-stabilizing factors are the microtubule-associated proteins (MAPs) [for a review see Avila et al., 1994].

Microtubule-associated protein 1B (MAP1B) is the first MAP expressed during neuron development [Bloom et al., 1985]. Moreover, MAP1B may be involved in axon development [DiTella et al., 1996], and this function may be regulated by MAP1B phosphorylation. Two main modes of MAP1B phosphorylation [Ulloa et al., 1993a] have been identified [Ulloa et al., 1993b]: mode I is catalyzed by proline-directed protein kinases, such as glycogen synthase kinase 3 (GSK3) or cyclin-dependent kinase 5 [Pigino et al., 1997; Goold et al., 1999], and mode II is catalyzed by non-proline-directed protein kinases such as casein kinase II [Ulloa et al., 1993b].

During axonogenesis, microtubule polymerization takes place, and newly assembled microtubules are characterized by the presence of tyrosinated α-tubulin [Tanaka et al., 1995]. The α-tubulin mRNA encodes a C-terminal tyrosine residue [Villasante et al., 1986], but once α-tubulin is incorporated into microtubules, tyrosine can be removed by a specific, although as yet unidentified, carboxypeptidase [Argarana et al., 1980]. When these microtubules are depolymerized, however, a tubulin-tyrosine ligase (TTL) can replace this tyrosine residue [Arce et al., 1978] at the C terminus of the α-tubulin monomer [Beltramo et al., 1987]. Thus, newly assembled microtubules, like those present during axonogenesis, are composed largely of tyr-tub (α-tubulin containing a C-terminal tyrosine) [Gundersen et al., 1984]. It is widely accepted that modifications of the neuronal cytoskeleton are required to allow the morphological changes required to develop their specific functions. Neuronal migration is one of the processes that occurs upon morphological transformation of neurons [Edmondson and Hatten, 1987; Gasser and Hatten, 1990; Rivas and Hatten, 1995]. Indeed, in vitro analyses of migrating neurons have shown that both microtubules and actin microfilaments undergo dynamic changes that are essential for neuronal movement [Rivas and Hatten, 1995]. In such a context, we have previously characterized a *Map1b* mutant mouse that displays several abnormalities in the structure of its nervous system [Gonzalez-Billault et al., 2000; Gonzalez-Billault et al., 2005]. These abnormalities are related to problems in neuronal migration, especially in the migration of neurons dependent on the extracellular matrix protein, Reelin [Gonzalez-Billault et al., 2005]. Additionally, MAP1B-deficient neurons display an imbalance in the content of tyrosinated and detyrosinated microtubules both in CNS and PNS neuronal cultures [Gonzalez-Billault et al., 2001; Gonzalez-Billault et al., 2002], suggesting that proper neuronal migration may be dependent on the dynamic properties of microtubules. To address this issue, we analyzed the possible interaction between MAP1B and TTL, which may have relevance to the dynamics of microtubule assembly and thus neuronal migration and axonogenesis.

Materials and Methods

Materials

The following antibodies were used: N19, against MAP1B (Santa Cruz Biotechnology); SM131, against phospho-MAP1B, mode I (Sternberger Monoclonals); YL1/2, against tyrosinated α-tubulin (Sigma), anti-β tubulin (Sigma), anti-TTL, ID3 (a kind gift of Dr. D. Job, Grenoble, France); 9E10 and A14, anti-c-myc (Santa Cruz Biotechnology). cDNA for TTL was from Dr. D. Job; cDNA for GSK3β, tagged at its C terminus with the myc epitope peptide, and the functional tet-responsive system, to express GSK3β, have been described [Lucas et al., 2001].

Cell Culture

N2A mouse neuroblastoma cells (ATCC CCL 131, American Type Culture Collection, Rockville, Md., USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics and incubated in a humidified atmosphere with 7% CO_2 [Jimenez-Mateos et al., 2005b].

Immunostaining

N2A cells grown on glass coverslips were incubated with phosphate-buffered saline (PBS)/0.1% (v/v) Triton X-100 for 5 min and then with PBS/5% bovine serum albumin (BSA) for 1 h. Subsequently, primary antibodies raised against the indicated proteins were diluted in PBS/1% BSA and used to label the cells. The secondary antibodies (anti-mouse lgGs) were from Molecular Probes (Eugene, Oreg., USA).

Pregnant wild-type or *Map1b* heterozygous females [Gonzalez-Billault et al., 2000; Gonzalez-Billault et al., 2002] were sacrificed at gestational day 18 and the embryos removed under sterile conditions; cortex slices were obtained and stained with an antibody raised against α-tyrosinated tubulin and with an antibody against total tubulin.

Immunoprecipitation

Cells were homogenized in 0.7 ml of cold immunoprecipitation buffer [1% (v/v) Triton X-100, 150 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF and 0.5% (v/v) Nonidet P40]. The homogenate was centrifuged at 1,600 *g* for 15 min at 4°C, and the supernatant was considered as the total cell lysate. Immunoprecipitation conditions were as described [Dotti and Banker, 1987]. Immunoprecipitated protein was characterized by Western blotting carried out as described [Gonzalez-Billault et al., 2001]. The Quality One (Bio-Rad, Hercules, Calif., USA) program was used for densitometric analysis.

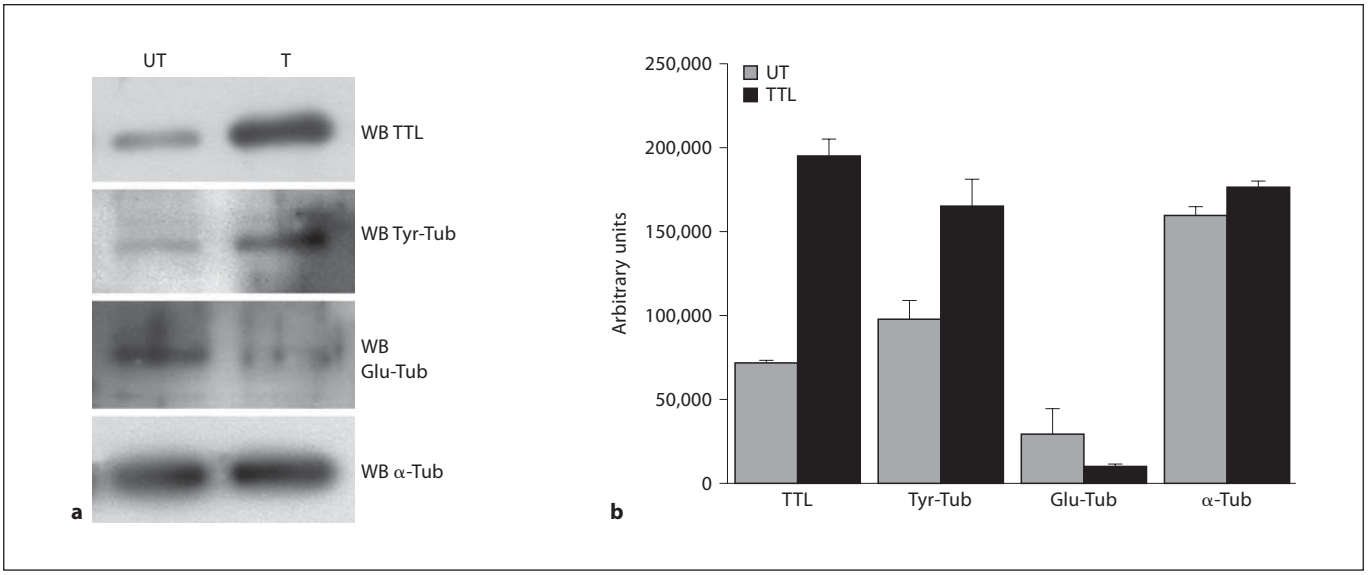

Fig. 1. Overexpression of TTL in neuroblastoma cells. **a** Western blots of extracts from control (UT) and TTL-transfected (T) cells. The specific antibody used is indicated to the right of each Western blot (WB). The lower panel indicates the overall levels of α-tubulin expression. **b** Quantitative analyses show that the increase in TTL expression is paralleled by an increase in the amount of tyrosinated tubulin. Gray and black bars correspond to control and TTL-transfected cells, respectively.

DNA Transfection

After 18 h in culture, N2A cells were transfected or cotransfected with cDNA constructs corresponding to the functional tet-responsive system to express GSK3β [Lucas et al., 1998] and with cDNA encoding TTL. Transfections were carried out using Lipofectamine reagent (Invitrogen). Cells were fixed at different times after transfection and processed for immunofluorescence staining.

Pull-Down Assay

Glutathione S-transferase (GST) and GST-TTL recombinant proteins were cloned in the pGEX-4T plasmid, and the proteins were expressed in *Escherichia coli* BL21 cells. Transformed cells were cultured in SOC medium (20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose), then grown in SOB medium (SOC without glucose). Recombinant protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C. The cell lysates [Jimenez-Mateos et al., 2005b] were centrifuged, and the supernatant (500 μg protein) was then incubated with 50 μl of glutathione-Sepharose 4B beads (Pierce) for 2 h at room temperature. This mixture was then incubated with brain or N2A protein extracts (500 μg protein). The beads were washed 4 times with buffer X, boiled at 95°C in X μl SDS-PAGE sample buffer, and the eluted proteins were subjected to SDS-PAGE.

Nocodazole Treatments

Control and MAP1B-deficient hippocampal neurons were cultured on glass coverslips covered with poly-D-lysine (Sigma). After 48 h in culture, when most of the cells reached stage III of development [Dotti et al., 1988], 10 μM nocodazole (final concentration) was added to the cultures. After 30 min the medium was replaced with medium lacking nocodazole, and the cells were fixed at different time points ranging from 0 to 60 min. To visualize tyrosinated microtubules, after nocodazole treatment the cells were stained at different time points with an antibody against α-tubulin. We used rhodamine-phalloidin for actin staining, allowing us to detect the shape and limits of individual neurons. Afterwards, we quantitated fluorescence intensity to establish the recovery of tyrosinated microtubules. For that purpose, the emitted fluorescence was attenuated with glass neutral density filters. Images were formed on the faceplate of the camera that was set manually for black level, gain and sensitivity. The images were then directly digitized into a Metamorph/Metafluor image processor controlled by a host PC (Universal Imaging, West Chester, Pa., USA). Fluorescence intensity was measured in axons from 20–30 neurons per time point, and the average intensity per axon was then calculated for each time point.

Results

Our purpose was to analyze whether one of the enzymes regulating the tyrosination/detyrosination cycle of α-tubulin, TTL, interacts with MAP1B. Therefore, we chose mouse neuroblastoma N2a cells as a working model due to their high level of MAP1B expression. We overexpressed a full-length cDNA encoding TTL in these cells, and extracts of transfected cells showed increased amounts of tyrosinated α-tubulin (fig. 1a; quantitation shown in fig. 1b). This increase in the levels of tyrosinat-

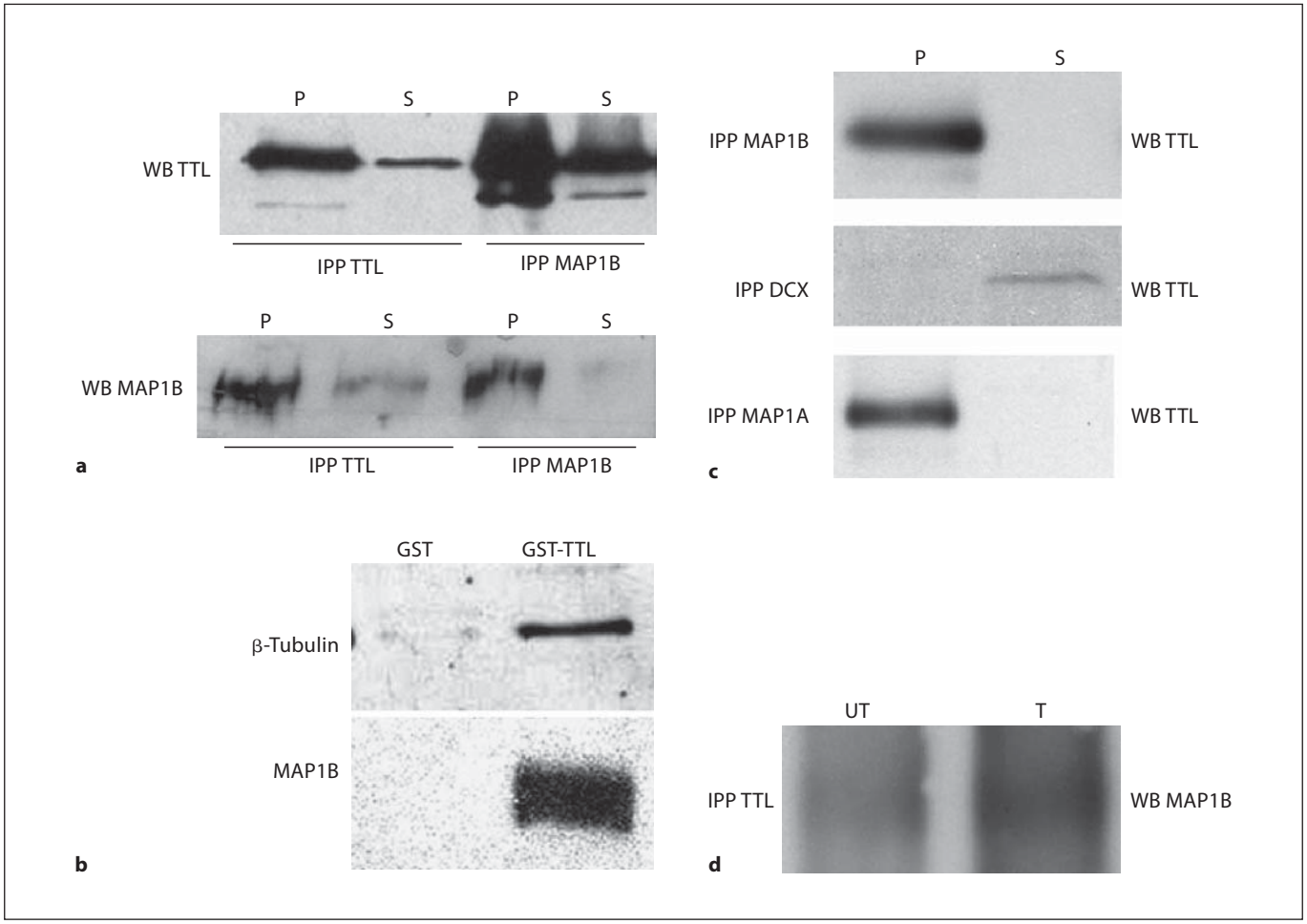

Fig. 2. Biochemical interaction between MAP1B and TTL. **a** TTL coimmunoprecipitates with MAP1B from brain extracts. Embryonic brain extracts were subjected to immunoprecipitation using an antibody against TTL (IPP TTL) or an antibody against MAP1B (IPP MAP1B). Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting (WB). The blot was probed with a polyclonal antibody against TTL (upper panel) and then with an antibody against MAP1B (lower panel). The protein present in the precipitate (P) or in the soluble fraction (S) is indicated. **b** MAP1B is recovered in a GST-TTL pull-down assay. Embryonic brain extracts were incubated with either GST or GST-TTL fusion protein. The recovery of β-tubulin served as a positive control of a known interaction with TTL (upper panel). **c** Brain extracts were immunoprecipitated with antibodies against the MAPs, MAP1B, doublecortin (DCX), and MAP1A. Proteins were visualized by Western blotting using anti-TTL. **d** Untransfected (UT) or transfected (T) N2a cells were incubated with anti-TTL, and the immunoprecipitated proteins were visualized by Western blotting with anti-MAP1B.

ed microtubules correlated with a decrease in the amount of detyrosinated microtubules. These variations in the amount of tyrosinated and detyrosinated tubulin did not reflect any variation in the overall levels of α-tubulin because no changes were detected using an antibody raised against total α-tubulin in both control and TTL-transfected cells (fig. 1a, WB α-tubulin panel). These experiments demonstrated that TTL-expressing N2a cells were indeed a good model to evaluate the dependence of MAP1B on TTL function.

To test whether MAP1B interacts with TTL, we performed immunoprecipitation of extracts derived from TTL-transfected cells. The immunoprecipitates obtained with anti-MAP1B or anti-TTL were enriched for TTL and MAP1B (fig. 2a). These results suggest that both MAP1B and TTL were able to reciprocally immunoprecipitate each protein, indicating that the enzyme was indeed interacting with MAP1B. To confirm these results, we used a pull-down approach. We generated a recombinant fusion protein, GST-TTL, that was then incubated with ex-

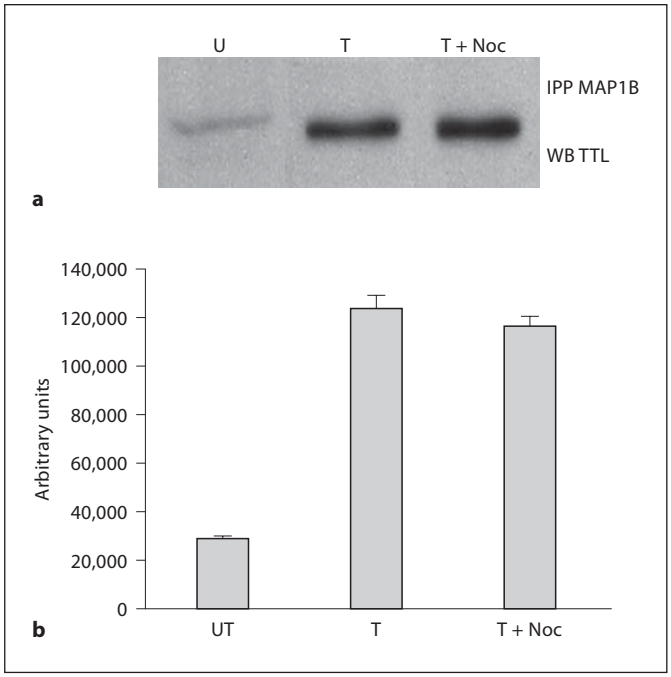

Fig. 3. Nocodazole does not affect the MAP1B-TTL interaction. **a** Extracts from TTL-transfected neuroblastoma N2a cells were immunoprecipitated with anti-MAP1B after treatment with nocodazole (T + Noc) or vehicle (T). **b** Quantitation of TTL recovered after immunoprecipitation with anti-MAP1B.

tracts of embryonic day 18 (E18) brain. GST-TTL was able to pull down MAP1B, whereas GST itself did not (fig. 2b). We used the binding of β-tubulin to GST-TTL as a positive control for our assay. To confirm that TTL interacts with MAP1B in brain, we performed immunoprecipitation assays using extracts of neurons. Indeed, anti-MAP1B immunoprecipitated TTL from neurons (fig. 2c). To test the specificity of this response, we performed immunoprecipitation with another MAP, namely doublecortin. Doublecortin was not able to immunoprecipitate TTL from brain extracts (fig. 2c). Interestingly, when we immunoprecipitated with anti-MAP1A (MAP1A is highly similar to MAP1B), TTL was again recovered in the pellet fraction (fig. 2c), suggesting that TTL interacts with MAP1 proteins in the region of high homology between MAP1A and MAP1B. Finally, we tested the MAP1B-TTL interaction by immunoprecipitating protein from N2a cells (control or transfected with TTL) and then analyzing the recovery of MAP1B. TTL was able to interact with MAP1B in untransfected cells, indicating that endogenous TTL was indeed bound to MAP1B (fig. 2d, UT). Additionally, MAP1B recovery was higher from extracts derived from TTL-transfected cells. It is possible that the interaction between TTL and MAP1B could be caused by a cross-linking effect of microtubules. Therefore, to test if TTL and MAP1B can interact directly, we immunoprecipitated TTL from untreated or nocodazole-treated cells (nocodazole is a microtubule-depolymerizing drug). Figure 3 indicates, however, that there were no differences in the amount of TTL recovered from cell extracts immunoprecipitated with anti-MAP1B in the absence or presence of 10 mM nocodazole for 30 min (fig. 3a, b).

Because TTL could interact with MAP1B, the next experiment was designed to analyze the functional consequences of MAP1B loss of function on TTL activity. Hippocampal neurons derived from control and MAP1B-deficient mice were cultured for 36 h over laminin, allowing them to reach the so-called stage III of development that is characterized by the presence of a distinguishable axon and several minor processes in culture [Dotti et al., 1988]. Then, microtubules were disassembled by nocodazole treatment for 30 min, after which the drug was removed and cells were incubated for different periods to allow microtubule repolymerization. In control neurons there was an increase in the amount of tyrosinated microtubules after nocodazole removal (fig. 4); this increase correlated with the prevalent localization of microtubules enriched in tyrosinated α-tubulin at the distal region of the axon, in a time-dependent manner. In MAP1B-deficient neurons, however, the level of tyrosinated α-tubulin microtubules decreased, and they had a most proximal axonal localization even at the later time points (1 h after nocodazole removal). To quantify the differences observed in our culture experiments, we used the Metamorph/Metafluor system to measure the fluorescence due to tyrosinated microtubules at the different time points after nocodazole removal (see Materials and Methods). Figure 5 confirms the results obtained by visual inspection, indicating that in the absence of MAP1B the recovery of tyrosinated microtubules was clearly slower, confirming that the absence of MAP1B decreases TTL activity.

To further test the effect of MAP1B on TTL function, we expressed TTL together with different constructs for MAP1B in a MAP1B-free context, such as COS-7 cells. We used the binary tetracycline transactivator system to express full-length MAP1B, the MAP1B heavy chain and the MAP1B light chain 1 (fig. 6). MAP1B heavy chain expression statistically increased up to 130% the amount of tyrosinated microtubules in COS-7 cells. The results for full-length MAP1B and MAP1B light chain 1 are also shown

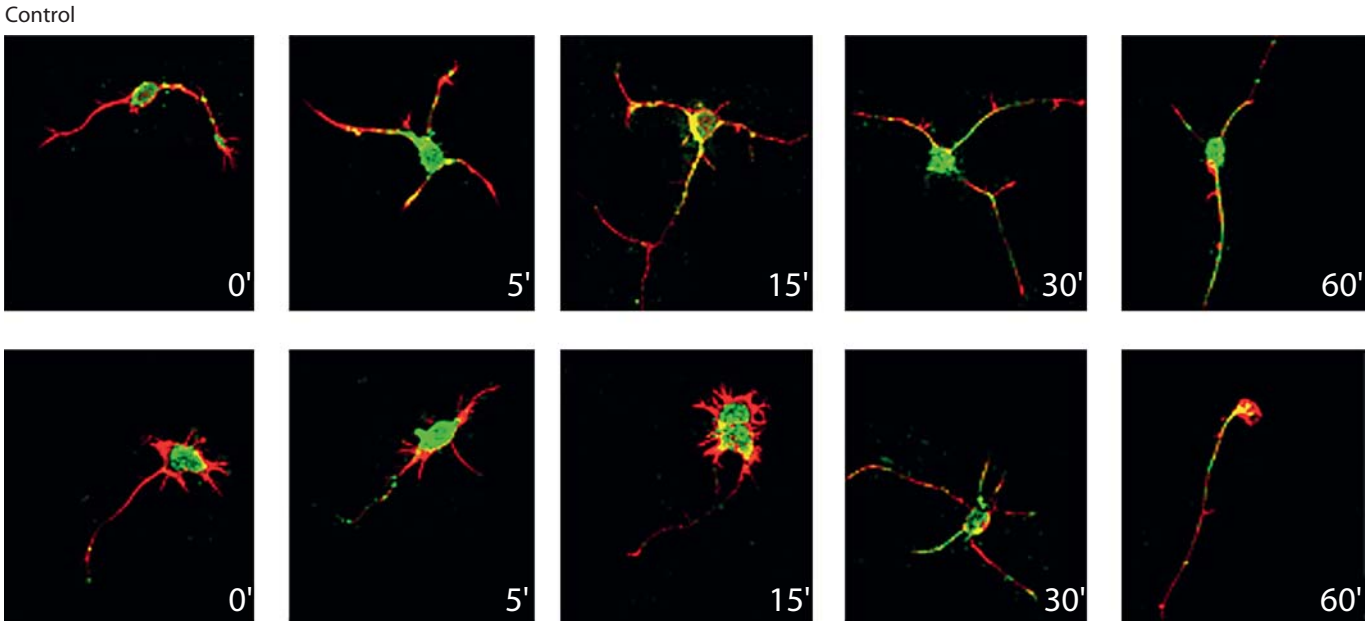

Fig. 4. Neurons lacking MAP1B display delayed recovery of tyrosinated microtubules after nocodazole treatment. The upper panel shows control neurons treated with nocodazole, allowed to recover for different periods, as indicated, and then fixed for immunofluorescence analysis. The neurons were stained with an antibody against tyrosinated tubulin (green) and rhodamine-phalloidin (red). Also, MAP1B-deficient (KO) neurons were incubated with nocodazole and then washed out to allow the recovery for microtubule assembly.

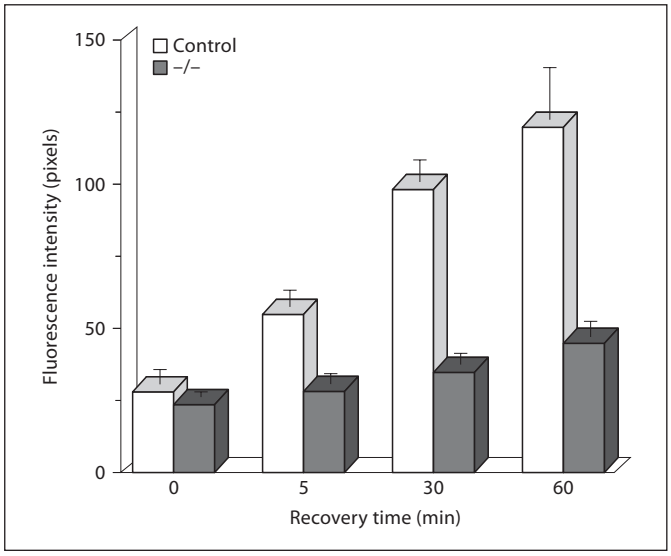

Fig. 5. Quantitative fluorescence of control and MAP1B-deficient cells. Using the Metamorph/Metafluor system, we quantitated the recovery of fluorescence due to tyrosinated tubulin in cells treated first with nocodazole and afterwards allowed to recover after the drug had been washed out. Note the significant differences among the control (white bars) and MAP1B-deficient (grey bars) groups.

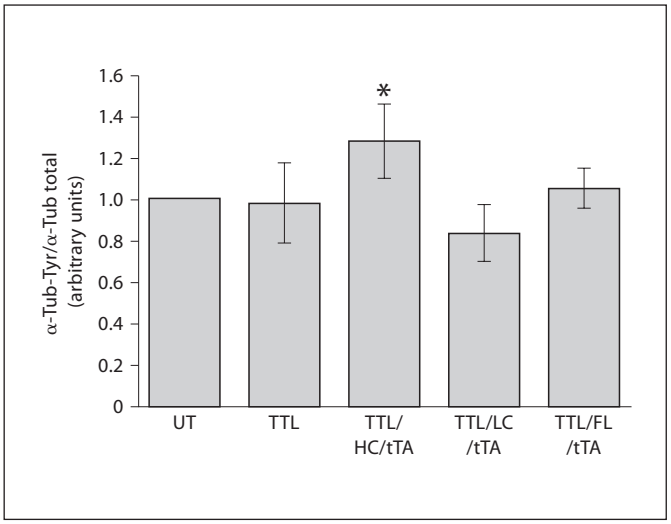

Fig. 6. MAP1B interaction with TTL in COS-7 cells induces an increase in the tyrosinated microtubules. The proportion of tyrosinated microtubules was determined by Western blotting using an antibody against tyrosinated tubulin. An antibody against total α-tubulin was used to quantitate total tubulin – as a loading control. The graph represents three independent experiments. HC = MAP1B heavy chain; LC = MAP1B light chain; FL = full-length MAP1B. * $p < 0.05$.

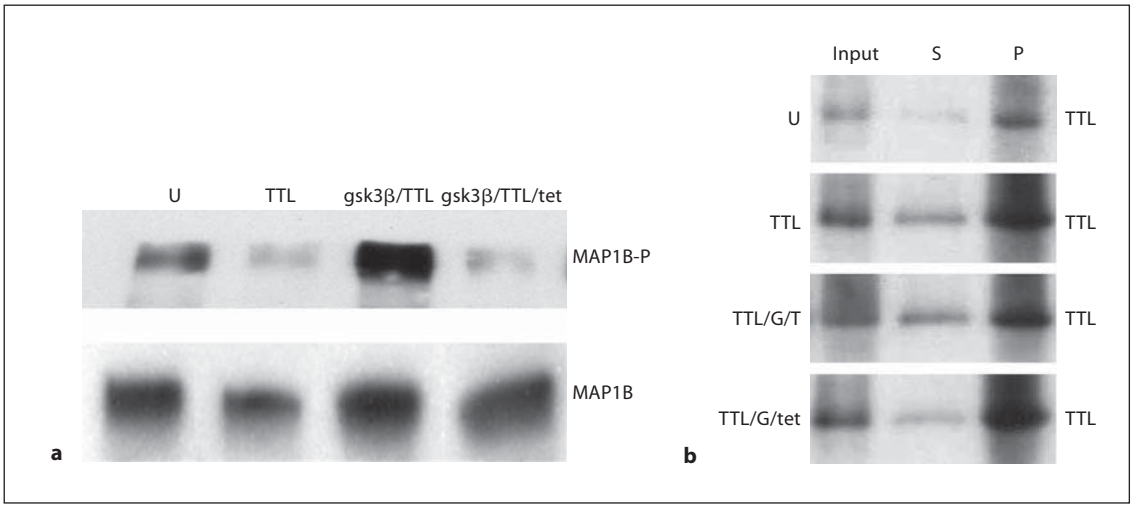

Fig. 7. The interaction between MAP1B and TTL is not regulated by MAP1B phosphorylation by mode I. **a** Western blots of protein extracts of control cells [untransfected (U)] and cells transfected with TTL (TTL), with TTL, gsk3β and tTA (gsk3β/TTL), or with TTL, gsk3β and tTA in the presence of tetracycline (gsk3β/TTL/tet). The primary antibodies used were against phospho-MAP1 (MAP1B-P) or against total MAP1B (MAP1B). **b** MAP1B coimmunoprecipitates with TTL from extracts of control cells [untransfected (U)] and cells transfected with TTL (TTL), TTL, gsk3β and tTA (TTL/G/T), or gsk3β and tTA in the presence of tetracycline (TTL/G/T tet). Proteins immunoprecipitated with the MAP1B phosphorylation-independent antibody NC19 were analyzed by Western blotting of the input (Input), supernatant (S) and pelleted (P) fractions using antibody ID3 against TTL.

and indicate no significant changes in the amount of tyrosinated α-tubulin after transfection. These results suggest that MAP1B heavy chain may enhance TTL activity.

Previous findings have indicated that GSK3β kinase phosphorylates MAP1B [Lucas et al., 1998; Goold et al., 1999], and loss of this activity correlates with an increase in the amount of detyrosinated microtubules. Additionally, neurons derived from a MAP1B mutant mouse also have higher amounts of detyrosinated microtubules, especially towards the distal parts of axons in cultures of both CNS and PNS neurons [Gonzalez-Billault et al., 2001; Gonzalez-Billault et al., 2002]. For this reason, we next tested whether the interaction between MAP1B and TTL is regulated by the phosphorylation state of MAP1B in N2a neuroblastoma cells. N2a cells were transfected with only a TTL-encoding plasmid or with that plasmid together with a binary expression system to induce GSK3β activity, here referred to as the G/T system (see Materials and Methods). Briefly, one plasmid encodes GSK3β having a c-myc tag and β-galactosidase cDNAs in opposite divergent orientations. Another plasmid encodes the tetracycline transactivator. Thus, the binding of the tetracycline transactivator to the regulatory regions of the plasmid encoding GSK3β and β-galactosidase will trigger the expression of both transgenes [Lucas et al., 2001]. To verify that the G/T system indeed caused an increase in GSK3β expression, we used anti-myc to reveal GSK3β overexpression. Myc-specific staining was found in cells transfected with the G/T expression system, and the increased GSK3β expression correlated with increased β-galactosidase expression in the nucleus, as expected (online suppl. fig. 1, www.karger.com/doi/10.1159/000109863). As a control the overexpression of both cDNAs was abolished when the cells were incubated in the presence of tetracycline (as expected for a Tet-Off-based system). Cell extracts were then prepared and analyzed by Western blotting (using an antibody raised against GSK3β). A protein, with a slightly decreased electrophoretic mobility, was only found in the G/T-induced system expressing myc-tagged GSK3β construct (online suppl. fig. 2, www.karger.com/doi/10.1159/000109863), with no apparent changes in the endogenous GSK3β expression (online suppl. fig. 2, www.karger.com/doi/10.1159/000109863; GSK3β). Also, a strong induction of the transgenes recognized by two different myc antibodies (online suppl. fig. 2, www.karger.com/doi/10.1159/000109863; anti-myc A14 and anti-myc 9E10) was found. In addition, we detected the expression of β-galactosidase only in extracts of cells transfected with the G/T system (online suppl. fig. 2, www.karger.com/doi/10.1159/000109863; β-galactosidase).

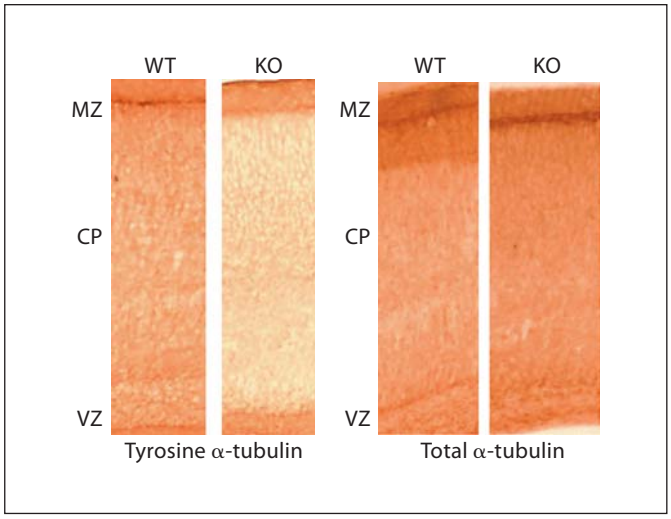

Fig. 8. Distribution of α-tyrosinated tubulin and total α-tubulin in brain cortical sections derived from wild-type and MAP1B-deficient mice. Serial sections of cerebral cortex were stained with antibodies against tyrosinated α-tubulin and total α-tubulin. In the case of tyrosinated tubulin there was a faint staining in the cortex of MAP1B-deficient animals (KO) as compared with wild-type littermates (WT). This decrease in the staining was found to be prominent in the marginal zone (MZ), cortical plate (CP) and ventricular zone (VZ) in the cortex. On the other hand, similar staining with an antibody that recognizes the total pool of α-tubulin indicates no differences in α-tubulin between WT and KO animals in all the aforementioned regions.

We then tested whether the increased GSK3β expression correlated with increased MAP1B mode I phosphorylation. MAP1B phosphorylated in mode I was greatly increased in the G/T system as recognized by the antibody SMI31 (fig. 7a, upper lane 'MAP1B-P'), and this increased phosphorylation was abolished in the presence of tetracycline, confirming that it was due to an increased GSK3β activity. The overall expression of MAP1B was unchanged under all conditions, as indicated by the phosphorylation-independent MAP1B antibody, NC19, (fig. 7a, lower panel 'MAP1B'). With this experimental paradigm we then analyzed whether the interaction between MAP1B and TTL may be regulated by the phosphorylation of MAP1B. We prepared extracts from neuroblastoma N2a cells transfected with TTL and GSK3β using the G/T system. Immunoprecipitation of MAP1B with antibody NC19 and subsequent Western blot analyses using antibody ID3 against TTL showed that in all the cases similar amounts of TTL were recovered (fig. 7b, compare lane P (pellet) among lanes TTL, TTL/GSK3β and TTL/GSK3β/tet). In some cases, there was slightly higher recovery of TTL after MAP1B immunoprecipitation of cells treated with tetracycline and a concomitant decrease in the soluble fraction (compare lanes S and P, TTL/GSK3β/tet). However, these differences were not significant (data not shown). These results suggest that although MAP1B is able to bind TTL, the binding and modulation of its activity may be independent of MAP1B phosphorylation by GSK3β.

Finally, to confirm that loss of MAP1B function indeed altered the in situ levels of α-tyrosinated tubulin, we performed immunohistochemical analyses in brain sections derived from control and MAP1B mutant embryos. Figure 8 shows serial cortical sections, from control and mutant E18.5 embryos, stained with an antibody that specifically recognizes α-tyrosinated tubulin (left panel, α-tyr tubulin) or with an antibody that recognizes all the α-tubulin isotypes (right panel, total α-tubulin). All sections shown in the figure correspond to the mediolateral cortex. Wild-type sections stained with anti-α-tyrosinated tubulin displayed robust staining in the cortical plate, as well as specific staining in the band of neurons corresponding to the marginal zone and in the ventricular zone. Tubulin staining was mostly restricted to the processes of neurons, rather than cell bodies. Notably, the MAP1B mutant cortical section showed reduced staining with anti-tyrosinated α-tubulin as compared with an equivalent control section. Despite this diminished staining, tyrosinated α-tubulin was enriched in the processes rather than in the cell bodies of neurons in the cortical plate, in the marginal zone and in the ventricular zone. These results are consistent with a role for MAP1B in the tyrosination/detyrosination cycle of α-tubulin in vivo. We then used an antibody that recognizes all α-tubulin forms as a control. For total α-tubulin, no differences were found in control and MAP1B-deficient brain sections, confirming that the relatively lower staining observed in MAP1B mutant sections was due to a specific decrease in tyrosinated α-tubulin. Taken together, these results indicate that loss of MAP1B is deleterious for the efficient tyrosination of microtubules both in vitro and in vivo and implicate this MAP in the regulation of tyrosination via TTL.

Discussion

During neuronal development, axonogenesis takes place through a process in which the distal (growing) part of the axon contains a higher proportion of newly assembled dynamic microtubules [Tanaka et al., 1995],

whereas the soma-proximal part contains a higher proportion of more stabilized microtubules. Newly assembled microtubules contain a higher proportion of the α-tubulin subunit, tyrosinated at its C-terminal end, than stable microtubules. Tubulin tyrosination takes place by the action of TTL [Arce et al., 1978; Erck et al., 2000]. On the other hand, it has been described that MAP1B is the first MAP to be expressed during nervous system development and can thus contribute to the stabilization and/or enhancement of microtubule polymerization [Bloom et al., 1985; Avila et al., 1994], which may control some of the dynamic properties of axon microtubules.

In this work, we have shown an interaction between MAP1B and TTL, and we have also described that overexpression of GSK3β, which phosphorylates MAP1B [Trivedi et al., 2005], but not that of TTL (which is a substrate for other kinases [Idriss, 2000]), does not dramatically change that interaction. Interaction between MAP1B and TTL facilitates the tyrosination of the α-tubulin subunit. This tyrosination mainly occurs in unpolymerized tubulin, which binds TTL at its β-subunit and facilitates the tyrosination of α-tubulin subunits [Wehland and Weber, 1987; Idriss 2000; Lafanechere and Job, 2000]. This mechanism probably cannot occur in the assembled microtubules because the TTL-binding site on β-tubulin subunits may be masked. However, it has been proposed that MAP1B prevents tubulin detyrosination in assembled microtubules [Goold et al., 1999]. Based on these results, we suggest that the prevention of assembled tubulin detyrosination may not be the function of MAP1B; rather, MAP1B may facilitate tyrosination of detyrosinated α-tubulin subunits.

The C-terminal tyrosine residue in α-tubulin can be enzymatically removed in microtubules by a carboxypeptidase activity that is associated with assembled microtubules, but this activity remains to be identified [Arce et al., 1978; Kumar and Flavin, 1981; Barra et al., 1988]. The reincorporation of tyrosine cannot occur, as previously indicated [Idriss, 2000], because TTL cannot properly bind to tubulin subunits in assembled microtubules. However, our data suggest that by interacting with MAP1B, which mainly binds to β-tubulin subunits [Avila et al., 1994], TTL may facilitate the incorporation of tyrosine into α-tubulin subunits. However, the nocodazole recovery experiments – in which a newly polymerized microtubule fraction was analyzed – suggested that this is not the case because cells lacking MAP1B also contain less newly assembled tyrosinated microtubules after release from the drug. Thus, we conclude that differences in the amount of tyrosinated microtubules may be attributed to differential tyrosination of α-tubulin due to the presence or absence of MAP1B.

Nevertheless, we cannot rule out other possibilities to explain how the interaction of TTL with MAP1B may result in an increase in the tyrosination of α-tubulin subunits [Goold et al., 1999]. Interestingly, another study conducted in a *Map1b* null mutant mouse indicated no changes in tyrosinated or detyrosinated microtubules in MAP1B-deficient dorsal root ganglia neurons [Bouquet et al., 2004]. This is not completely unexpected because the phenotype of such a mutant is quite subtle as compared with the hypomorphic transgenic line used in our study, raising the possibility that differences in the tyrosination and detyrosination dependence on the MAP1B-TTL interaction may also be affected by genetic background. We have previously shown that genetic background may modify MAP1B functional compensation by other proteins, such as EB1 [Jimenez-Mateos et al., 2005b].

On the other hand, we have preliminary evidence that a MAP1A-TTL interaction could occur, although at the present we do not know if the consequences of that relation could be similar to those of MAP1B-TTL interaction.

The functional consequences of changes in the dynamic properties of the neuronal microtubule and actin cytoskeleton are reflected in both in vitro and in vivo studies. Thus, pioneer studies using coculture of neurons and glial cells have shown that microtubules and actin microfilaments are required for neuronal movement in vitro [Edmondson and Hatten, 1987; Rivas and Hatten, 1995]. These studies have suggested the establishment of the leading process – a cytoplasmic extension of neurons that drives the movement of a migrating neuron – that may be involved in controlling cytoskeletal dynamics during movement. Subsequently, several in vivo studies have pointed out a role for the neuronal cytoskeleton in the control of neuronal migration. In those studies the loss of function of MAPs [Hirotsune et al., 1998; Gonzalez-Billault et al., 2000; Gonzalez-Billault et al., 2005] or the in utero overexpression of actin-regulating proteins [Kawauchi et al., 2003] was shown to disrupt normal neuronal migration. Interestingly, a gene-targeting approach to inactivate TTL gave rise to animals with blurred organization of cortical plate neurons and a disrupted corticothalamic loop [Erck et al., 2005]. These two aspects of CNS development have also been described to occur in MAP1B-deficient mice [Del Rio et al., 2004; Gonzalez-Billault et al., 2005], suggesting that the TTL-MAP1B interaction, which we describe here, may have a role in both

neuronal migration and axonal guidance. Additionally, it has recently been shown that tyrosination of microtubules is required for the interaction of CAP-Gly microtubule plus-end proteins (such as CLIP170 or p150^{glued}) with microtubules [Peris et al., 2006]. This could be part of the mechanisms involved in controlling neuronal shape, leading to abnormal neuronal outgrowth.

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References

Arce CA, Hallak ME, Rodriguez JA, Barra HS, Caputto R (1978): Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino acids on tubulin assembly. J Neurochem 31:205–210.

Argarana CE, Barra HS, Caputto R (1980): Tubulinyl-tyrosine carboxypeptidase from chicken brain: properties and partial purification. J Neurochem 34:114–118.

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

Barra HS, Arce CA, Argarana CE (1988): Post-translational tyrosination/detyrosination of tubulin. Mol Neurobiol 2:133–153.

Beltramo DM, Arce CA, Barra HS (1987): Tubulin, but not microtubules, is the substrate for tubulin:tyrosine ligase in mature avian erythrocytes. J Biol Chem 262:15673–15677.

Bloom GS, Luca FC, Vallee RB (1985): Microtubule-associated protein 1B: identification of a major component of the neuronal cytoskeleton. Proc Natl Acad Sci USA 82:5404–5408.

Bouquet C, Soares S, von Boxberg Y, Ravaille-Veron M, Propst F, Nothias F (2004): Microtubule-associated protein 1B controls directionality of growth cone migration and axonal branching in regeneration of adult dorsal root ganglia neurons. J Neurosci 24:7204–7213.

Del Rio JA, Gonzalez-Billault C, Urena JM, Jimenez EM, Barallobre MJ, Pascual M, Pujadas L, Simo S, La Torre A, Wandosell F, Avila J, Soriano E (2004): MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance. Curr Biol 14:840–850.

DiTella MC, Feiguin F, Carri N, Kosik KS, Caceres A (1996): MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth. J Cell Sci 109:467–477.

Dotti CG, Banker GA (1987): Experimentally induced alteration in the polarity of developing neurons. Nature 330:254–256.

Dotti CG, Sullivan CA, Banker GA (1988): The establishment of polarity by hippocampal neurons in culture. J Neurosci 8:1454–1468.

Edmondson JC, Hatten ME (1987): Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. J Neurosci 7:1928–1934.

Erck C, Frank R, Wehland J (2000): Tubulin-tyrosine ligase, a long-lasting enigma. Neurochem Res 25:5–10.

Erck C, Peris L, Andrieux A, Meissirel C, Gruber AD, Vernet M, Schweitzer A, Saoudi Y, Pointu H, Bosc C, Salin PA, Job D, Wehland J (2005): A vital role of tubulin-tyrosine-ligase for neuronal organization. Proc Natl Acad Sci USA 102:7853–7858.

Gasser UE, Hatten ME (1990): Neuron-glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation. J Neurosci 10:1276–1285.

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, Del Rio JA, Urena JM, Jimenez-Mateos EM, Barallobre MJ, Pascual M, Pujadas L, Simo S, Torre AL, Gavin R, Wandosell F, Soriano E, Avila J (2005): A role of MAP1B in Reelin-dependent neuronal migration. Cereb Cortex 15:1134–1145.

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, 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.

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.

Gundersen GG, Kalnoski MH, Bulinski JC (1984): Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently in vivo. Cell 38:779–789.

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.

Idriss HT (2000): Phosphorylation of tubulin tyrosine ligase: a potential mechanism for regulation of alpha-tubulin tyrosination. Cell Motil Cytoskeleton 46:1–5.

Jimenez-Mateos EM, Paglini G, Gonzalez-Billault C, Caceres A, Avila J (2005a): End binding protein-1 (EB1) complements microtubule-associated protein-1B during axonogenesis. J Neurosci Res 80:350–359.

Jimenez-Mateos EM, Wandosell F, Reiner O, Avila J, Gonzalez-Billault C (2005b): Binding of microtubule-associated protein 1B to LIS1 affects the interaction between dynein and LIS1. Biochem J 389:333–341.

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.

Kumar N, Flavin M (1981): Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin. J Biol Chem 256:7678–7686.

Lafanechere L, Job D (2000): The third tubulin pool. Neurochem Res 25:11–18.

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.

Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, Avila J (2001): Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J 20:27–39.

Mitchison T, Kirschner M (1988): Cytoskeletal dynamics and nerve growth. Neuron 1:761–772.

Peris L, Thery M, Fauré J, Saoudi Y, Lafanechère L, Chilton JK, Gordon-Weeks P, Galjart N, Bornens M, Wordeman L, Wehland J, Andrieux A, Job D (2006): Tubulin tyrosination is a major factor affecting the recruitment of CAP-Gly proteins at microtubule ends. J Cell Biol 174:839–849.

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.
Rivas RJ, Hatten ME (1995): Motility and cytoskeletal organization of migrating cerebellar granule neurons. J Neurosci 15:981–989.
Tanaka E, Ho T, Kirschner MW (1995): The role of microtubule dynamics in growth cone motility and axonal growth. J Cell Biol 128: 139–155.
Trivedi N, Marsh P, Goold RG, Wood-Kaczmar A, Gordon-Weeks PR (2005): Glycogen synthase kinase-3beta phosphorylation of MAP1B at Ser1260 and Thr1265 is spatially restricted to growing axons. J Cell Sci 118: 993–1005.
Ulloa L, Avila J, Diaz-Nido J (1993a): Heterogeneity in the phosphorylation of microtubule-associated protein MAP1B during rat brain development. J Neurochem 61:961–972.
Ulloa L, Diaz-Nido J, Avila J (1993b): Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells. EMBO J 12:1633–1640.
Villasante A, Wang D, Dobner P, Dolph P, Lewis SA, Cowan NJ (1986): Six mouse alpha-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. Mol Cell Biol 6:2409–2419.
Wehland J, Weber K (1987): Tubulin-tyrosine ligase has a binding site on beta-tubulin: a two-domain structure of the enzyme. J Cell Biol 104:1059–1067.