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MAP1B regulates microtubule dynamics by sequestering EB1/3 in the cytosol of developing neuronal cells

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Editor: Karin Dumstrei

1st Editorial Decision 26 July 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

While there is an interest in the paper, significant concerns are also raised with it that I am afraid preclude publication here. As you can see below, many issues are raised concerning the conclusiveness of the findings reported. In particular it is not clear if overepression or loss of MAP1b affects microtubule numbers, which could provide an alternative explanation for the results observed. Other issues are raised as well. Referee #3 also finds that some further insight into how MAP1B/EB interaction is regulated in space and time would be needed for publication here. Given these comment and as we only accept papers which receive enthusiastic support upon initial review, I am afraid that we can not offer to publish it here.

I thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1

Overall, this is a clearly written paper that covers a basic, yet important topic that will be interesting to both the developmental and neuronal biology fields. MAP1B and EBs are ubiquitous proteins that are used to establish microtubule (MT) arrays in every cell type. However, their unique importance

in establishing polarized MTs in neurons makes their protein-protein interactions and mechanisms of MT regulation particularly interesting in the developing neurons.
In this article the authors show that MAP1B has an effect on EB1/3 binding and EB1/3 regulation of microtubule (MT) growth. Through live animals, cell culture, and in vitro systems the authors succeed in demonstrating that MAP1B is necessary for restricting EB1/3 binding to the MT plus end. The authors suggest MAP1B achieves this by a two methods: excluding EBs from the MT lattice and sequestering EBs in the cytosol. While the former mechanism is a well-known function of MAP1B the latter is a novel method of EB regulation in neurons.
Major results include that with a loss of MAP1B, either through shRNA knockdown or mutant Map1b, there is a delocalization of EBs from the MT plus tip, echoed by other +TIPS, causing a change in MT dynamics that includes a decrease in number of dynamic plus ends, an increase in the plus end growth rate, and a loss of plus end pausing. The authors are also able to demonstrate that loss of MAP1B activity has the ability to abolish correct growth cone extension, an important developmental effect. However, the authors do not demonstrate that the effects on microtubule growth and EB protein behavior are direct. As well as defects at the plus end, cells depleted of MAP1B have a global reduction of microtubules. This reduction in microtubule number could influence the amount of free EB proteins and tubulin, and this could result in changes in plus end behavior. The manuscript would be much stronger if this issue could be addressed. I have included one suggestion below for an experiment that would more directly test the sequestration model. An alternative might be to show that depletion of a different MAP that is abundant in these cells has does not influence EB3 behavior.

Specific Comments:
1. In Figure 1C: why is the MAP1B staining seen primarily in one quadrant of the cell? Is this typical?

2. MAP1B staining in Figure 1 is quite punctate, but the GFP-tagged version smoothly lines microtubules. Based on other images is the difference due to expression level, antibody, or something else?

3. The authors' both over-express, via a GFP labeled construct, and knockdown, via shRNA, MAP1B in order to understand its effect on EB1/3. (Figure 2)
a. In Fig. 2, the two insets of panel A are not well labeled.
b. From Figure 2A the conclusion is drawn that "MAP1B-GFP localized both in the cytosol and along the MT lattice, without causing significant MT stabilization or bundling (Figure 2A)." The figure shows staining of a single cell. This is an important point for interpreting the paper. My interpretation of the same picture would be that because there are fewer EB1 comets, the microtubules are more stable, ie overall longer microtubules and so fewer growing plus ends. Microtubule stability, or different readouts of it, should be directly analyzed. Is there more acetylated tubulin? Alternately, the conclusion that microtubule stability is not affected should be tempered or removed.

4. shRNA knockdown of MAP1B also causes a reduction of dynamic MT plus ends but also a lengthening of EB comet length and an overall loss of MT density. (Figure 3). Overall this looks nice, but the idea that there are fewer MTs overall introduces some major caveats into the interpretation that MAP1B directly influences plus end behavior. The significance of longer comets is not clear?

5. The authors then turn in vitro experiments to establish how MAP1B and EB1 are interacting within cells, suggesting that the EBs and MAP1B directly bind. (Figure 4)
a. The conclusion from this figure is overstated: "In our assays, although both GFP-tagged versions of EBs did co-immunoprecipitate with MAP1B, GFP-EBs immunoprecipitated better with MAP1B than EBs-GFP, suggesting that the interaction is favored when EBs are not localized at MT plus-ends." Isn't it at least equally likely that the C-terminal tagging more directly interferes with forming a complex that includes MAP1B?
b. Another overstated conclusion from this data is: "Thus, the interaction between MAP1B and EBs occurs in cultured neuronal cells as well as in vivo." The experiment shows that MAP1B isolated from brain can interact with purified GST-EBs. It does not show that the two proteins interact in the neurons.
c. I am wondering why it was not possible to co-IP endogenous Map1B and EB1. It would be

helpful to at least mention the explanation. Also, it looks like nocodazole treatment only increases the EB3-MAP1B interaction, but not the EB1-MAP1B interaction. Is this correct? Any ideas about this point?

6. A FRAP assay helps to demonstrate that without MAP1B modulates EB3 mobility, especially when there are no MT plus ends to bind to. (Figure 5)
The FRAP data in Fig 5 showing that EB3 recovers more quickly when MAP1B is depleted is nice evidence for an in vivo interaction. The nocodazole experiment is a good idea.

7. Figure 6 is important, and shows nice effects of MAP1B on MT dynamics. However, again I am worried that the alterations in MT behavior might be secondary effects from the reduction in MT number/density. Is there more free tubulin in the cells that biases MT dynamics towards growth and reduces catastrophe? Could increased free tubulin or more EB3 per growing plus end result in increased velocity? The idea that there is more EB3 along the side of MTs in MAP1B-depleted cells seems important. Is it possible to quantitate this? Is it possible for example to take the ratio of EB3 on the MT to free EB3 in the cytosol?

8. I am wondering whether it is possible to directly test the sequestration model? No data on colocalization of EB3 and MAP1B is presented, so it would be great to have some other way to demonstrate cytoplasmic sequestration. In Figure 4 it is shown that the N-term of MAP1B is responsible to interacting with EB3. Can this region of MAP1B be targeted to mitochondria (or some other structure)? If so you might see EB3 concentrated there. If the phenotype of MAP1B depletion really arises from sequestration of EB proteins, this targeted construct might also be expected to rescue knockdown of MAP1B. My guess is that it may not fully rescue, but it may give an idea of which part of the MAP1B effect is due to altering EB behavior.

9. Figures 7 and 8 examine primary cultures of neurons from wt and MAP1B mutant mice. This is a nice addition. My major concern here is again how much of the MT phenotype is due to a direct link between MAP1B and EB proteins, and how much is secondary to general defects in the MT cytoskeleton? The discussion brings up this problem: "The reduced amount of growing MTs would increase the levels of soluble tubulin, displacing the equilibrium towards MT polymerization and in this way accelerating MT growth." However, the issue of whether the effect on EB behavior is direct or due to this more global effect on MT number is not addressed.

10. I had some trouble with the description of Figure 8. How is it possible for velocity of growth to be the same if EB3 comets move faster? Perhaps this section could be clarified? (Note that really in all figures it is speed that is being measured and not velocity as direction is not taken into account- I understand that in the field velocity is often used...)

11. The difference in comet behavior in wt and mutant hippocampal neurons shown in the movies is really quite striking. The back up in EB3-GFP along microtubules is also extremely striking- again I think it might be important to try to quantitate this as it seems like it may be a very important part of the phenotype.

Referee #2

In this manuscript the authors investigated the relationship of two EBs (1 and 3) and the microtubule associated protein MAP1b. The authors claim a direct interaction of these two EBs with MAP1b and conclude that "MAP1b locally controls MT dynamics via its regulation of EB3 during axonogenesis" and propose the "existence of a new mechanism of EB1/3 regulation by MAP1b that contributes to orchestrate MT dynamics during neuronal differentiation".

Comments:

1) An interaction between MAP1b and EBs is deduced from IPs only, but the authors do not investigate directly the binding domains and possible regulating mechanisms. There is no mechanistic approach other than transfecting or silencing MAP1b.

2) The title of the manuscript is not supported by the data, particularly the aspect of the "axonal

cytoplasm":
a. Almost the entire study has been conducted in neuroblastoma cells devoid of axons.
b. Axogenesis is not addressed. The scarce data shown for axons only represents one stage of axogenesis, not the process.
c. Fig. 4a clearly shows that MAP1b does not interact with EB1 (IP), in all immunofluorescent figures there is a pronounced difference in the localization of EB1/3 and MAP1b, indicating that these proteins do not interact (contrary to the main claim of the paper).

3) The authors overexpress or silence MAP1b, but do not investigate the effect on the number of microtubules, which is likely to change after expression of any MAP. As a matter of fact, ALL effects induced by modulating MAP1b levels (comet number, comet length etc.) can be explained by a change in microtubule numbers, and also by altered microtubule stability. For example, Fig. 3D clearly shows that silencing of MAP1b results in fewer microtubules.

4) Important controls for the dynamics of microtubules have not been conducted. Other MAPs, taxol and nocodazole (in suitable concentrations) controls are missing.

5) The authors skip from EB1 to EB3 for no good reason, and Figure 4 actually shows that the interaction of EB1 and 3 with MAP1b is very different. But instead of employing this difference to clarify the influence, authors just neglect EB1 from this point onwards.

6) It is not clear how the quantification of comet numbers, brightness and comet lengths has been conducted, this needs to be explained in detail.

7) Data is often conflicting, but these conflicts are not explained, investigated or discussed. In detail:
a. Fig. 2 states that MAP1b overexpression reduces the number of comets, Fig. 3 states that silencing of MAP1b does the same. This suggests a problem in the quantification, since it is clear both from the pictures in these Figures but also in the quantification (Fig. 2D) that the intensities of the comets change, which needs to be taken into consideration.
b. In Fig. 9 authors depict that MAP1b binds EB1 and 3 while this is not supported by colocalization images and by the IP in Fig. 4a.

8) In Fig. 7, 1DIV is not a suitable age, as the size of the growth cone and the axonal length is not comparable between wt and MAP1bKO (1.5-2 fold difference). Accordingly, ALL the parameters measured are approximately changed by 1.5-2 fold, hence this might simply be a concentration/convolution effect.

9) Figure legends are too brief, descriptions of what the reader has to look at are missing, scale bars are missing and panels are confused (e.g. Fig. 8 H and D...)

10) The model is only partially supported by the data. The different size of the growth cones and the axons are problematic, binding of EB3 to MAP1b might be true, but not of EB1. Looping might be the result of changed growth cone size / delayed outgrowth.

In general, there is a mismatch between statements in the text and what the figures show. Also, the Discussion is not related to the data presented. Thus the reader is left uncertain whether this manuscript will shed light on the processes regulating MT dynamics. A more rigorous interpretation of the data is recommended.

Referee #3

The paper describes an unexpected interplay between microtubule accessory proteins MAP1B and EB1/EB3 during neuron extension and thereby proposes a new function of MAP1B as a direct regulator of the activities of EB proteins. The authors show that MAP1B interacts directly with both EB1 and EB3 in the cytosol and in vitro; binding to MAP1B is shown to affect the localization, mobility and dynamics of the EBs. The molecular interaction has not been characterised in detail but the effects of where GFP is located were checked to avoid artifacts.

Since the focus is mainly on the effects in neurons, what I felt to be missing is some idea of how the

cell controls the interactions, how the proteins are made to behave differently at different times and in different regions of a neuron. Both MAP1B and EB1 are known to be subject to phosphorylation, which affects their interactions with MTs. Does it also directly regulate their interaction with each other? Information could be gained both in vitro and in cells by expressing phospho-protein mimics and/or phosphorylation-resistant proteins.
The secondary effect on CLIPs does not need to be included in the final model (fig. 9); it just distracts attention from the primary interaction.

Additional Correspondence 10 August 2012

Thank you for your email regarding the editorial decision taken on your manuscript (EMBOJ-2012-82396). I have now had a chance to take a look at the points you raise.

One of the main issues raised with the paper is that we need stronger support for that the effect of MAP1b on microtubule dynamics is mediated via binding to EB1/3 and not via global effects on MT numbers. If you can add data to further support the sequestration model then I can offer to look at a resubmission. You would also have to address the other issues raised as well. It would also be good if you could add some data on how the MAP1B/EB1 interaction is regulated. We don't need the full mechanism, but some insight into this issue would clearly strengthen the paper.

I should add that for resubmissions that we consider the novelty at time of submission and if needed might involve new referee(s).

Resubmission received 14 January 2013

Response to reviewers

Referee #1:

Overall, this is a clearly written paper that covers a basic, yet important topic that will be interesting to both the developmental and neuronal biology fields. MAP1B and EBs are ubiquitous proteins that are used to establish microtubule (MT) arrays in every cell type. However, their unique importance in establishing polarized MTs in neurons makes their proteinprotein interactions and mechanisms of MT regulation particularly interesting in the developing neurons. In this article the authors show that MAP1B has an effect on EB1/3 binding and EB1/3 regulation of microtubule (MT) growth. Through live animals, cell culture, and in vitro systems the authors succeed in demonstrating that MAP1B is necessary for restricting EB1/3 binding to the MT plus end. The authors suggest MAP1B achieves this by a two methods: excluding EBs from the MT lattice and sequestering EBs in the cytosol. While the former mechanism is a wellknown function of MAP1B the latter is a novel method of EB regulation in neurons. Major results include that with a loss of MAP1B, either through shRNA knockdown or mutant Map1b, there is a delocalization of EBs from the MT plus tip, echoed by other +TIPS, causing a change in MT dynamics that includes a decrease in number of dynamic plus ends, an increase in the plus end growth rate, and a loss of plus end pausing. The authors are also able to demonstrate that loss of MAP1B activity has the ability to abolish correct growth cone extension, an important developmental effect. However, the authors do not demonstrate that the effects on microtubule growth and EB protein behavior are direct. As well as defects at the plus end, cells depleted of MAP1B have a global reduction of microtubules. This reduction in microtubule number could influence the amount of free EB proteins and tubulin, and this could result in changes in plus end behavior. The manuscript would be much stronger if this issue could be addressed. I have included one suggestion below for an experiment that would more directly test the sequestration model. An alternative might be to show that depletion of a different MAP that is abundant in these cells has does not influence EB3 behavior.

Reviewer 1 provided several interesting suggestions and comments that we considered very

useful to make our manuscript more conclusive. We have made our best to answer every point raised by him/her, as shown below:

-One of the main concerns raised by referee 1 was that he/she found we had not demonstrated that the effects of MAP1B on EBs localization are direct and not mediated by the effects of MAP1B on the MT lattice. We have addressed this important concern by using both of the experimental approaches suggested by him/her:
a) depletion of a different MAP that is also abundant in these cells, tau,
b) and an experiment to test the sequestration model.

See a detailed explanation in specific answers below.

Specific Comments:

1. In Figure 1C: why is the MAP1B staining seen primarily in one quadrant of the cell? Is this typical?

Actually, this is not typical and therefore it might not be the best example to show. Thus, we changed the picture in Figure 1C and included a more representative one of MAP1B staining.

2. MAP1B staining in Figure 1 is quite punctate, but the GFP-tagged version smoothly lines microtubules. Based on other images is the difference due to expression level, antibody, or something else?

The referee is right in that MAP1B staining shows a more punctate staining that the GFPversion of the protein. In overexpressing cells, total amount of MAP1B is increased, most likely allowing it to bind more smoothly along MTs. Also, we observed that MAP1B staining is somewhat heterogeneous, presenting a clear MT pattern in some cells and a more diffuse staining in others, most probably depending on cell state. This is in line with previous reports in which a large soluble cytoplasmic pool of MAP1B has been described in brain and cultured neurons. Therefore, the differences in staining are most likely due to both expression levels and antibody staining, as well as to the particular state of the cell.

3. The authors' both over-express, via a GFP labeled construct, and knockdown, via shRNA, MAP1B in order to understand its effect on EB1/3. (Figure 2)
a. In Fig. 2, the two insets of panel A are not well labeled.
Correct labeling has been included accordingly.
b. From Figure 2A the conclusion is drawn that "MAP1B-GFP localized both in the cytosol and along the MT lattice, without causing significant MT stabilization or bundling (Figure 2A)." The figure shows staining of a single cell. This is an important point for interpreting the paper. My interpretation of the same picture would be that because there are fewer EB1 comets, the microtubules are more stable, ie overall longer microtubules and so fewer growing plus ends. Microtubule stability, or different readouts of it, should be directly analyzed. Is there more acetylated tubulin? Alternately, the conclusion that microtubule stability is not affected should be tempered or removed.

Figure 2A shows a representative field of MAP1B-GFP transfected cells in which EB1 comets clearly disappear (note two other transfected cells on the sides of the pictures, outlined in yellow). To address the reviewer comment, cells expressing MAP1B-GFP were stained with antibodies that recognized stable or total MTs and fluorescence intensity was measured in non transfected and transfected cells (Figure 2E and F). We found that cells expressing MAP1BGFP did not show significant changes in MT density or stability, confirming that MAP1B is a weak MT stabilizer as shown before. As an extra control for the specificity of MAP1B action on EBs, we ectopically expressed tau-GFP and analyzed its effects on the localization of EBs and on MTs (Figure S1). We selected cells expressing similar expression levels of both MAPs (low to medium), avoiding cells expressing high levels of tau protein that clearly induces MT bundling. Cells expressing moderate levels of tau-GFP do not show either an increased number

of MTs in our cells and only present a slight raise in MT stability (Figure S1D. Interestingly, EB comets display a normal pattern in tau-GFP expressing cells (Figure 2A,B and C). Hence, we conclude that displacement of EB proteins from MTs in MAP1B transfected cells is not due to alterations in MT numbers or stability and are specific for MAP1B.

4. shRNA knockdown of MAP1B also causes a reduction of dynamic MT plus ends but also a lengthening of EB comet length and an overall loss of MT density. (Figure 3). Overall this looks nice, but the idea that there are fewer MTs overall introduces some major caveats into the interpretation that MAP1B directly influences plus end behavior. The significance of longer comets is not clear?

To answer to this point, we generated N1E-115 cell lines stably deficient in tau protein, another classical neuronal MAP that is abundantly expressed in these cells (Figure S2A). Tau-knockeddown
cells show a decrease in MT density more dramatic to the one present in MAP1Bdeficient cells (Figure 3G and Figure S2D). Importantly, binding of EBs to MTs is not increased in tau-depleted cells. This further confirms the fact the enhanced accumulation of EB1/3 on MT plus-ends and along MT stretches present upon MAP1B depletion is not due to the reduction in the amount of MTs and points to a direct influence of MAP1B on EB behavior.

5. The authors then turn in vitro experiments to establish how MAP1B and EB1 are interacting within cells, suggesting that the EBs and MAP1B directly bind. (Figure 4)
a. The conclusion from this figure is overstated: "In our assays, although both GFP-tagged versions of EBs did co-immunoprecipitate with MAP1B, GFP-EBs immunoprecipitated better with MAP1B than EBs-GFP, suggesting that the interaction is favored when EBs are not localized at MT plus-ends." Isn't it at least equally likely that the C-terminal tagging more directly interferes with forming a complex that includes MAP1B?

The referee is right in that C-terminal tagging could somewhat impair the interaction between MAP1B and EBs. We therefore rephrased the sentence accordingly.

b. Another overstated conclusion from this data is: "Thus, the interaction between MAP1B and EBs occurs in cultured neuronal cells as well as in vivo." The experiment shows that MAP1B isolated from brain can interact with purified GST-EBs. It does not show that the two proteins interact in the neurons.

c. I am wondering why it was not possible to co-IP endogenous Map1B and EB1. It would be helpful to at least mention the explanation

To address points b and c, we performed immunoprecipitations of endogenous MAP1B and EB3 in mouse embryonic brain, confirming that both proteins form a complex *in vivo*. This new result is incorporated in Figure 4B. Some explanatory text was also included and the conclusion was rephrased accordingly.

In addition, we have observed that MAP1B binds more efficiently with EB3 than with EB1. This, together with the fact that the interaction might be labile, has most likely hampered us to succeed with the co-IP of endogenous MAP1B and EB1 proteins

Also, it looks like nocodazole treatment only increases the EB3-MAP1B interaction, but not the EB1-MAP1B interaction. Is this correct? Any ideas about this point?

The referee is right since it seems that only the interaction of MAP1B with EB3 is increased but not with EB1. We have rephrased the sentence accordingly. Actually, MAP1B binds more weakly with EB1 than with EB3 and therefore this interaction might be more labile.

6. A FRAP assay helps to demonstrate that without MAP1B modulates EB3 mobility, especially when there are no MT plus ends to bind to. (Figure 5) The FRAP data in Fig 5

showing that EB3 recovers more quickly when MAP1B is depleted is nice evidence for an in vivo interaction. The nocodazole experiment is a good idea.

We are grateful to the referee and we indeed consider this experiment quite conclusive showing that MAP1B regulates EB3 mobility and dynamics in the cytosol of differentiating neuronal cells, especially when EB3 is localized in the cytosol.

7. Figure 6 is important, and shows nice effects of MAP1B on MT dynamics. However, again I am worried that the alterations in MT behavior might be secondary effects from the reduction in MT number/density. Is there more free tubulin in the cells that biases MT dynamics towards growth and reduces catastrophe? Could increased free tubulin or more EB3 per growing plus end result in increased velocity? The idea that there is more EB3 along the side of MTs in MAP1B-depleted cells seems important. Is it possible to quantitate this? Is it possible for example to take the ratio of EB3 on the MT to free EB3 in the cytosol?

We agree with the referee in that we cannot rule out that the increase in MT growth speed could be a mix of the result of an increase in free tubulin in the cytosol, due to the reduction in MT number and the enhanced accumulation of EB3 on MT plus-ends and MT stretches. However, reduction in MT numbers is limited to account for this significant increase in growth speed. Moreover, as mentioned, tau depletion leads to a more prominent reduction in MT numbers and does not enhance EB3 accumulation at MT plus-ends. We have included some comments on this issue in the Discussion section. We tried to perform the quantification of the ratio between cytosolic and microtubular EB3-GFP in control and MAP1B-depleted cells. However, although we took cells with similar expression levels of EB3-GFP, we found some technical difficulties since in some cells EB3-GFP mostly accumulated at MT plus-ends and in others it was found along MT segments.

8. I am wondering whether it is possible to directly test the sequestration model? No data on colocalization of EB3 and MAP1B is presented, so it would be great to have some other way to demonstrate cytoplasmic sequestration. In Figure 4 it is shown that the N-term of MAP1B is responsible to interacting with EB3. Can this region of MAP1B be targeted to mitochondria (or some other structure)? If so you might see EB3 concentrated there. If the phenotype of MAP1B depletion really arises from sequestration of EB proteins, this targeted construct might also be expected to rescue knockdown of MAP1B. My guess is that it may not fully rescue, but it may give an idea of which part of the MAP1B effect is due to altering EB behavior.

In Figure S3C we have now included some pictures in which some partial colocalization of MAP1B and EB3 is shown in cells treated with Nocodazole at concentrations that depolymerize MTs and both proteins are totally cytosolic. Furthermore, as suggested by the referee, we analyzed the effects of a construct encoding an N-terminal fragment of MAP1B (aa 1-508) on EB1 localization (gift of Dr. Propst). This MAP1B deletion lacks the microtubule binding domain and shows diffuse cytoplasmic distribution and no effect on microtubules (our own results and Tögel et al., 1998). Moreover, our in vitro pull-down assays indicate that this MAP1B fragment interacts directly with both EBs. Importantly, in cells expressing this deletion fragment of MAP1B, EB1 comets mostly disappear and the protein remains sequestered in the cytosol (Figure S5). These results support our point that MAP1B sequesters EBs in the cytosol. Rescue experiments were not possible because of technical problems (transfection of MAP1Bdeficient cells with this construct didn´t work properly).

9. Figures 7 and 8 examine primary cultures of neurons from wt and MAP1B mutant mice. This is a nice addition. My major concern here is again how much of the MT phenotype is due to a direct link between MAP1B and EB proteins, and how much is secondary to general defects in the MT cytoskeleton? The discussion brings up this problem: "The reduced amount of growing MTs would increase the levels of soluble tubulin, displacing the equilibrium towards MT polymerization and in this way accelerating MT growth." However, the issue of whether the effect on EB behavior is direct or due to this more global effect on MT number is not addressed.

To address this point we made use of tau-deficient neurons obtained from tau-knockout mouse strain available in our lab. tau-KO neurons present a delay in axon growth and a reduction in the amount of MTs similar to the ones observed in our MAP1B-deficient neurons. Therefore, if the effects of MAP1B on EBs localization were just due to its effects on MTs, EB comet pattern in tau-KO neurons should be similar. Since the effects of MAP1B deficiency were more prominent on EBs, mainly in growth cones, we analyzed EB3 comet pattern in growth cones of tau-/- neurons. Our results indicate that the length of EB3 comet does not change significantly in growth cones of tau-deficient neurons. These data support that the effects of MAP1B on EB3 localization in neurons are not due to the reduction of MT numbers. These new results are presented in Figure S10C.

10. I had some trouble with the description of Figure 8. How is it possible for velocity of growth to be the same if EB3 comets move faster? Perhaps this section could be clarified? (Note that really in all figures it is speed that is being measured and not velocity as direction is not taken into account- I understand that in the field velocity is often used...)

We agree in that this figure is quite complex. We have tried to explain it in a clearer way in the Results section. As suggested, we have changed velocity by speed, although in the field both words are usually interchangeable.

11. The difference in comet behavior in wt and mutant hippocampal neurons shown in the movies is really quite striking. The back up in EB3-GFP along microtubules is also extremely striking- again I think it might be important to try to quantitate this as it seems like it may be a very important part of the phenotype.

As suggested, we have quantified EB3-GFP direction of movement in wt and MAP1B-deficient neurons and results are presented in Figure 8C. Indeed, MAP1B-deficient neurons present an increased number of backwards displacements of EB3-GFP comets.
Overall, we believe that we have made every effort to answer to all the points raised by the reviewer as we have performed a whole set of diverse and new experiments, ranging from transfections, generation of tau–deficient stable cell lines, culture of tau-KO neurons, immunoprecipitations, as well as thorough quantifications.

Referee #2

In this manuscript the authors investigated the relationship of two EBs (1 and 3) and the microtubule associated protein MAP1b. The authors claim a direct interaction of these two EBs with MAP1b and conclude that "MAP1b locally controls MT dynamics via its regulation of EB3 during axonogenesis" and propose the "existence of a new mechanism of EB1/3 regulation by MAP1b that contributes to orchestrate MT dynamics during neuronal differentiation".

Comments:

1) An interaction between MAP1b and EBs is deduced from IPs only, but the authors do not investigate directly the binding domains and possible regulating mechanisms. There is no mechanistic approach other than transfecting or silencing MAP1b.

We show a direct interaction *in vitro* between MAP1B and EB1/3 by pull-down assays (Fig.4D) and, in this revised version, we also show partial colocalization of MAP1B and EB3 in the cytosol upon Nocodazole treatment (Figure S3C). By FRAP assays, we demonstrate that this interaction is functional neuronal cells, (Figure. 5). Furthermore, we confirm most of our findings in primary neurons from hypomorphous Map1b-/- mice. We show that the N-terminal region of MAP1B interacts with EB proteins (Figure. 4D). In this revised version, we provide data about the regulation of the interaction between MAP1B and EBs by phosphorylstion (Figure S4).

2) The title of the manuscript is not supported by the data, particularly the aspect of the "axonal cytoplasm":
a. Almost the entire study has been conducted in neuroblastoma cells devoid of axons.
b. Axogenesis is not addressed. The scarce data shown for axons only represents one stage of axogenesis, not the process.
We have changed the title of the manuscript accordingly. Now it is entitled**: MAP1B regulates microtubule dynamics by sequestering EB1/3 in the cytosol of developing neuronal cells**

Our study only deals with one stage of axonogenesis, stage 3 in primary hippocampal neurons, because this has been the one that has been reported to be modulated by MAP1B.

c. Fig. 4a clearly shows that MAP1b does not interact with EB1 (IP), in all immunofluorescent figures there is a pronounced difference in the localization of EB1/3 and MAP1b, indicating that these proteins do not interact (contrary to the main claim of the paper).

As mentioned in the manuscript, we consistently got EB1 in our immunoprecipitates, although the amount was much smaller than of EB3, and light EB1 bands are present in co-IP assays in Fig.4A (compare with GFP lanes that present a much higher expression). Moreover, GST-EB1 was pulled-down with MAP1B from brain lysates and with MAP1B-6x-His-tag (Fig. 4C and D). In the case of EB3, we also showed that the interaction is functional by FRAP assays, and now we included new data indicating that both proteins form a complex in mouse brain (see Figure 4B). Moreover, we have now included pictures in which a partial colocalization of MAP1B and EBs is found, and this colocalization is enhanced upon cell treatment with Nocodazole, which release MAP1B and EBs from MTs (Figure S3C). These data support our biochemical and FRAP findings that indicate that the interaction is enhanced in the cytosol. In addition, cells were fixed with Methanol (+PFA) in order to visualize EBs comets properly. It is therefore likely that diffusion of EB proteins (which are not big) would preclude a better colocalization with MAP1B in the cytosol.

3) The authors overexpress or silence MAP1b, but do not investigate the effect on the number of microtubules, which is likely to change after expression of any MAP. As a matter of fact, ALL effects induced by modulating MAP1b levels (comet number, comet length etc.) can be explained by a change in microtubule numbers, and also by altered microtubule stability. For example, Fig. 3D clearly shows that silencing of MAP1b results in fewer microtubules.

In this revised version of the manuscript, we have quantified total MT density as well as MT stability in cells in which MAP1B levels are either increased or decreased. We found that overexpression of MAP1B-GFP at moderate levels does not induce significant changes in MT number or stability (Figure 2E and F), in line with reports that point at MAP1B as a weak MT stabilizer. in the case of, MAP1B depletion, it in deed leads to a decrease in MT density (Figure 3E and 3G). However, in this revised version we have used another neuronal MAP that exerts similar effects on MTs as a control. If all the effects of MAP1B on EBs were mediated by its actions on MTs, then modulating tau levels should have similar effects on EBs. However, this was not the case, since tau depletion does not enhance EBs binding to MTs (Figure 3H and S2B). These new data confirm that the actions of MAP1B on EBs are specific for this MAP and not mediated by its action on the MT network.

4) Important controls for the dynamics of microtubules have not been conducted. Other MAPs, taxol and nocodazole (in suitable concentrations) controls are missing.

In the new Figure S3, we show the effects of Nocodazole treatments on the MT network as well as on EBs and MAP1B localization in N1E-115 cells. Moreover, as mentioned above, we have tested and compared the effects of MAP1B and another MAP, tau, on EBs localization and MT number in our working models (new Figures S1, S2 and S10C).

5) The authors skip from EB1 to EB3 for no good reason, and Figure 4 actually shows that the interaction of EB1 and 3 with MAP1b is very different. But instead of employing this difference to clarify the influence, authors just neglect EB1 from this point onwards.

Although we started our study with both EB1 and EB3, we decided to primarily focus on EB3 from Figure 4 on, because we consistently found that the interaction between MAP1B and EB3 was more prominent. Moreover, while EB1 is ubiquitous, EB3 is highly enriched in brain, mainly in neurons, as MAP1B, and we found that at a functional level, this interaction might be more relevant. We agree with the referee in that the difference in the interaction may be of interest and this could be the subject of further studies.

6) It is not clear how the quantification of comet numbers, brightness and comet lengths has been conducted, this needs to be explained in detail.

Quantifications were performed in every case using ImageJ software. A more detailed explanation has been included in the Materials and Methods section.

7) Data is often conflicting, but these conflicts are not explained, investigated or discussed. In detail:
a. Fig. 2 states that MAP1b overexpression reduces the number of comets, Fig. 3 states that silencing of MAP1b does the same. This suggests a problem in the quantification, since it is clear both from the pictures in these Figures but also in the quantification (Fig. 2D) that the intensities of the comets change, which needs to be taken into consideration.
We do not consider these results are conflicting. The reduction in EB comet density is in each case a result of different causes. In MAP1B-depleted cells the number of MTs is reduced and therefore the amount of EB comets that mark growing MTs. However, in MAP1B overexpressing cells, the number of MTs is not significantly altered. For this reason, the reduction in the amount of EB comets is not due to changes in MT density but is most likely a result of the displacement of EBs from MTs. EBs displacement could arise from both an excess of MAP1B on MTs –that may hinder low affinity binding sites for EBs- and sequestration in the cytosol by diffuse MAP1B. We further corroborated the sequestration model with new data included in new Figure S5. We overexpressed an N-terminal fragment of MAP1B that does not interact with MTs (MAP1B 1-508-Myc). This MAP1B fragment displaces EB1 from MT plusends and sequesters it in the cytosol.

b. In Fig. 9 authors depict that MAP1b binds EB1 and 3 while this is not supported by colocalization images and by the IP in Fig. 4a.

As shown in Figure 4A, in transfected cells the amount of expressed GFP is always much higher than that of EB1. However, GFP is not present in a complex with MAP1B but EB1 is. This supports the fact that although the amount of EB1 in the complex is always very small as compared to the amount of EB3, the interaction with MAP1B is genuine. CoIP assays of overexpressed proteins as well as pull-down assays (Fig.4A and 4C and D)) show that both EB1 and EB3 are in a complex with MAP1B. Moreover, in this revised version, we have included data that show a partial colocalization of MAP1B with EB3 in Nocodazole-treated cells (Fig. S3C) and new coIP data that show that endogenous MAP1B and EBs interact in embryonic mouse brain (Fig. 4B).

8) In Fig. 7, 1DIV is not a suitable age, as the size of the growth cone and the axonal length is not comparable between wt and MAP1bKO (1.5-2 fold difference). Accordingly, ALL the parameters measured are approximately changed by 1.5-2 fold, hence this might simply be a concentration/convolution effect.

In the past, we showed that the lack of MAPB induces a delay in axon outgrowth that is more prominent at the beginning of the axonogenesis process in hippocampal neurons (1DIV) (Gonzalez Billault et al., Mol Biol Cell, 12, 2087-2098). In the current study, one of our aims was to clarify how MAP1B controls microtubule dynamics at plus-ends during axon outgrowth

and growth cone advance. Therefore, we undertook our studies in 1DIV wt and MAP1Bdeficient neurons that showed the biggest differences in axon length and growth cones size.

9) Figure legends are too brief, descriptions of what the reader has to look at are missing, scale bars are missing and panels are confused (e.g. Fig. 8 H and D...).
We took a further look at these points made by the reviewer, corrected the panels or text that could be confusing, and added some extra explanatory text in some figure legends.

10) The model is only partially supported by the data. The different size of the growth cones and the axons are problematic, binding of EB3 to MAP1b might be true, but not of EB1. Looping might be the result of changed growth cone size / delayed outgrowth. In general, there is a mismatch between statements in the text and what the figures show. Also, the Discussion is not related to the data presented. Thus the reader is left uncertain whether this manuscript will shed light on the processes regulating MT dynamics. A more rigorous interpretation of the data is recommended.

We find that our model is just a summary of the presented data. We do not consider that different axon and growth cone sizes are a problem since to our knowledge no direct correlation between axon length/growth cone size and EB binding to microtubules has been described. Moreover, tau-KO neurons also present shorter axons than wt ones but they do not present longer EB3 comets. We disagree in that the Discussion is not related to the data presented. Interpretations and discussion of the data are always arguable but we consider that we have carefully discussed all our findings. Moreover, we trust that the whole set of new results that we have included in this revised version of the manuscript will help clarify all the aspects that could not be totally clear in the previous one.

Referee #3

The paper describes an unexpected interplay between microtubule accessory proteins MAP1B and EB1/EB3 during neuron extension and thereby proposes a new function of MAP1B as a direct regulator of the activities of EB proteins. The authors show that MAP1B interacts directly with both EB1 and EB3 in the cytosol and in vitro; binding to MAP1B is shown to affect the localization, mobility and dynamics of the EBs. The molecular interaction has not been characterised in detail but the effects of where GFP is located were checked to avoid artifacts.

Since the focus is mainly on the effects in neurons, what I felt to be missing is some idea of how the cell controls the interactions, how the proteins are made to behave differently at different times and in different regions of a neuron. Both MAP1B and EB1 are known to be subject to phosphorylation, which affects their interactions with MTs. Does it also directly regulate their interaction with each other? Information could be gained both in vitro and in cells by expressing phospho-protein mimics and/or phosphorylation-resistant proteins. The secondary effect on CLIPs does not need to be included in the final model (fig. 9); it just distracts attention from the primary interaction.

We agree with this referee in that understanding how the interaction between MAP1B and EBs is regulated is of interest. Since the focus of this manuscript is the interaction between MAP1B and EBs and the modulation by MAP1B of EBs localization, mobility and action on MT dynamics, we found no much more space to explore how the interaction is regulated. Addressing this issue would be the subject of another full article. Indeed, this submitted manuscript is already quite extensive. Anyway, we did some experiments to get an idea about whether phosphorylation might play a role in the regulation of the MAP1B/EBs interaction. As suggested by the reviewer, we used a (GSK-3) phosporylation-resistant MAP1B mutant (gift of Dr. Gordon-Weeks) and confirmed that upon transfection in N1E-115 cells, EB1 is also displaced from MTs, which is in line with an interaction of MAP1B and EBs when MAP1B is

not phosphorylated by GSK-3. Since these were isolated data and didn´t fit well in the manuscript, we have not included them but we could do it if required. Moreover, since MAP1B interaction with MTs in neurons has been shown to be modulated by different kinases, we considered that the use of pharmacological inhibitors could give us more information about the possible regulation of the interaction of MAP1B with EBs by phosphorylation. Based on this, we used different pharmacological inhibitors of some of these kinases, such as the prolinedirected kinases GSK-3 and Cdk5, or the non-proline-directed kinase CK2 or JNK and checked whether the interaction between MAP1B and EB3 was altered. We found that inhibition of both GSK-3 or Cdk5 increased this binding, suggesting that the interaction between MAP1B and EB3 is regulated by these proline-directed kinases. These new results are included in new Figure S4. Further experiments to fully clarify the regulation of the interaction will be done for future works, but we hope this is enough to give a hint about phosphorylation as being a key mechanism of regulation of the MAP1B/EBs interaction, as suggested by the reviewer.

In addition, we have modified figure 9 according to the referee´s comment and eliminated CLIPs from the final scheme.

2nd Editorial Decision 18 February 2013

Thank you for submitting your manuscript to the EMBO Journal, which is a resubmission of MS 82396. Your study has now been re-reviewed by the original referees #1 and 3. Referee #2 was not available to review this submission.

As you can see below, both referees appreciate the added data and find that the conclusion that MAP1B regulates plus end dynamics via sequestrating EB1/3 in the cytosol is well demonstrated. Given these comments, I would therefore like to invite you to submit a suitably revised manuscript. Referee #1 has a minor remaining suggestion that I would like to you to take into consideration in a final revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

The additional analysis and inclusion of tau for comparison has made the paper much stronger. The authors now have much more data to support their model that MAP1B influences EB proteins and plus end dynamics through sequestration in the cytosol, rather than an indirect mechanism. They argue that their data supports a model in which MAP1B lowers the effective concentration of EB proteins in the cell that are free to interact with the plus end. While I am now much more convinced by their data, I would like to ask one more question: Can this idea be supported by overexpression of EB1? This would be predicted to mimic the MAP1B knockdown phenotype if their model is correct.

There are still some parts of the paper that are not carefully written, only a couple are specified below, but I would suggest a careful read-through:

Section 1 in results title: "MAP1B localizes along EB1/3 positive growing MTs" I didn't see data to support this title in this section- MAP1B and EB proteins are seen in the same part of the cells, but I did not see any evidence or description that they can really be seen clearly on the same MT.

Some typos, eg ciclin instead of cyclin

Referee #3

The authors have made significant improvements to this interesting report of an unexpected activity

of MAP1B in sequestration of EB1/3. It remains unknown how this activity is controlled in turn, to vary the effect of EB1/3 on MT dynamics at different times in different regions, but I agree that this should be a topic for later research.

2nd Revision - authors' response 05 March 2013

Response to reviewer 1:

Referee #1

-The additional analysis and inclusion of tau for comparison has made the paper much stronger. The authors now have much more data to support their model that MAP1B influences EB proteins and plus end dynamics through sequestration in the cytosol, rather than an indirect mechanism. They argue that their data supports a model in which MAP1B lowers the effective concentration of EB proteins in the cell that are free to interact with the plus end. While I am now much more convinced by their data, I would like to ask one more question: Can this idea be supported by overexpression of EB1? This would be predicted to mimic the MAP1B knockdown phenotype if their model is correct.

To answer this remaining question, we have performed transfection experiments in which N1E-115 cells were transfected with GFP-tagged EB1. EB1-GFP accumulated at MT plus-ends, when expressed at low levels. However, when EB1-GFP was expressed at medium to high levels, this +TIP bound to MT segments and even along the MT lattice. This constitutes a different way of increasing EB1 effective concentration in cells, thus mimicking the MAP1B knockdown phenotype, as predicted by the referee. These new results are included in the paper as a new supplemental figure (Figure S2).

-There are still some parts of the paper that are not carefully written, only a couple are specified below, but I would suggest a careful read-through:

Section 1 in results title: "MAP1B localizes along EB1/3 positive growing MTs" I didn't see data to support this title in this section- MAP1B and EB proteins are seen in the same part of the cells, but I did not see any evidence or description that they can really be seen clearly on the same MT.
We have changed the title of this Section accordingly to: "MAP1B and EB1/3 localize in neurites and growth cones of differentiating neuronal cells".

Some typos, eg ciclin instead of cyclin

This and other typos were amended. Overall, we have had a careful read-through the text and corrected the found mistakes.

We would like to thank once more the reviewer for the constructive and useful suggestions provided.

