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Myelin suppresses axon regeneration by PIR-B/SHP-mediated inhibition of Trk activity

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 November 2010

The referees appreciate that the findings provide novel molecular insight into how MAG inhibits neurite outgrowth. However they also find that some additional work is needed in order for further consideration here. In particular all three referees find that some further in vivo analysis is needed to support the PIRB/TrkB/SHP link. This would include the need for PIR-B loss of function analysis to test if optic nerve regeneration and TrkB phosphorylation levels are affected under such conditions. Should you be able to address the concerns raised, then we would consider a revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the major issues at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Fujita et al showed their data suggesting a role of SHP in mediating the inhibitory activity of myelin associated glycoprotein (MAG) on axon growth and perhaps regeneration. Although many pieces of biochemical data are present to indicate that MAG triggers the formation of a protein complex which consists of Pir-B, TrkB and SHP1/2, most of these experiments are done with over-expressed systems and do not reflect the in vivo scenario. In many cases, there are insufficient data to support the claims made by the authors.

Other comments:

Is MAG's effect dependent on TrkB? What is the contribution of different receptor components such as Nogo-66 receptor, P75 and others?

Does endogenous PirB interact with TrkB with MAG?

How about the expression of SHP1/2 in CNS neurons (different ages)? CGNs are relatively immature and other adult neurons such as retinal ganglion cells should be assessed.

In the experiments shown in Fig. 5, what percentages of SHP1/2 are inhibited by siRNA? How long does the silencing effect last? Both mutant mice are available and should be used to verify the conclusion. Even silencing of SHP could promotes axon regeneration, this is not sufficient to claim the contribution of PirB/TrkB/SHP. In particular, the authors just published data showing no regeneration of corticospinal axons in Pir-B knockout mice.

Referee #2 (Remarks to the Author):

This manuscript by Fujita et al hypothesizes that the PIR-B MAG receptor inhibits axonal regeneration by recruiting the Trk phosphatases SHP-1 and SHP-2 to TrkB, resulting in a downregulation of Trk activity and regeneration. The biochemistry in general supports this hypothesis; (1) PIR-B and TrkB co-immunoprecipitated, (2) MAG stimulated PIR-B and TrkB association, (3) MAG stimulated SHP association with TrkB and this required PIR-B, (4) MAG decreased TrkB tyrosine phosphorylation and this required SHP, and (5) MAG could not suppress TrkB phosphorylation from PIR-B knockout neurons. SHP and SHP association with TrkB was also required for the ability of MAG to suppress neurite outgrowth. A key experiment shows that suppression of SHP by siRNA infusion in vivo stimulated axonal regeneration after optic nerve injury in mice.

In general, I very much liked this paper. The biochemical and neurite outgrowth data are quite novel and convincing, with a few minor issues. Where the data needs strengthening is the in vivo data of figure 5 (see point 1). With those additional experiments, the paper will make a very important contribution to the axon degeneration and neurotrophin fields.

Major comment:
1. Figure 5. The regeneration experiment shows only that SHP1 and 2 are required for regeneration of the injured optic nerve. While this is a novel finding, it says nothing about whether PIR-B or TrkB are involved in the SHP siRNA effects. Cytokines that also use SHPs regulate survival and regeneration. Therefore, the following experiments should be performed:
A. The authors have the PIR-B knockouts in hand, and an effective way to deliver siRNA to the injured optic nerve. They should show that PIR-B downregulation or knockout results in enhanced regeneration of the optic nerve, as they have shown for SHP-1/2 knockdown.
B. They should perform immunoflourescence with anti-phospho-TrkB to show that SHP and PIR-B knockdown enhances TrkB activity.

Minor comments:
3. Figure 1G. Which of the two bands is PIR-B? It is running as a tight doublet in the IP, and not in the lysate blot.

4. Fig 2A, 2D. Both SHP-2 and PIR-B levels increase in the plus MAG lane, so I was not convinced that MAG addition increased PIR-B associations with SHP-2. This blot should be quantified. In 2D, the SHP2 band in the minus MAG/plus TrkB lane is uneven, and I am not convinced that SHP2-TrkB associations are increasing with MAG addition.

5. Fig. 3B, C, D and 4A. A general issue with the data in these figures is that SHP-1 siRNA or inhibitor decreases TrkB tyrosine phosphorylation and neurite outgrowth in the absence of MAG. Shouldn't it increase TrkB tyrosine phosphorylation if the authors' hypothesis is correct?

6. Fig. 4A or 4D. It should be shown that either SHP siRNA or the SHP/TrkB association blocking peptide increase TrkB phosphorylation.

Referee #3 (Remarks to the Author):

This is a very solid manuscript, detailing a pathway from MAG to PirB to SHP1-2 to TrkB dephosphorylation as a mechanism for MAG inhibition of neurite growth and regeneration. A few revisions and a couple of additional experiments would greatly strengthen the manuscript.

In Fig 1F, I don't see a convincing difference in the TrkB blot after MAG-Fc treatment. For review, please show a series of biological replicates of this data that make a convincing statement of PirB-TrkB association promoted by MAG treatment, or else revise the point in the results/discussion.

In Fig 2C, the increase in SHP2 pulled down by TrkB does not appear significant given the increase in TrkB pulled down in the IP. For review, please show a series of biological replicates of this data that make a convincing statement of SHP2-TrkB association promoted by MAG treatment, or else revise the point in the results/discussion.

The results section (page 6) refers to SHP2 being pulled down into a TrkB-PirB complex as shown in Figs. 2C-D, but these data only show SHP2-TrkB association, not "complex" formation with PirB. The wording of the results (and throughout) should be revised to reflect the data.

The loss of neurite growth down to 40 um average in Fig. 4B-C may not reflect the specific importance of TrkB inhibition in MAG-induced neurite inhibition, but rather the general importance of TrkB to neurite growth, below which nothing can inhibit further (e.g. if the 40 um represents a sprouting or adhesion fundamentally different from neurite elongation). This point should be discussed in the results and discussion as an alternative interpretation of those data.

In Figs. 4F-G, SHP-Trk peptide appears to inhibit the basal levels of neurite growth. Can the authors explain how that fits their model?

What is the effect of PirB knockout on optic nerve regeneration? These experiments should be performed and included in the paper.
What is the percent transfection of the CGNs? Of the retinal cells in vivo? Can this be assessed by immunostaining?

The number of axons regenerating in the optic nerve is exceedingly small, even if statistically significant. This should be discussed in the results and discussion section.
Supplemental Fig 1A and B can probably be squeezed into the regular manuscript, avoiding the need to use supplemental data.

1st Revision - authors' response 13 January 2011

Responses to the reviewers' comments

Reviewer #1

1. Although many pieces of biochemical data are present to indicate that MAG triggers the formation of a protein complex which consists of Pir-B, TrkB and SHP1/2, most of these experiments are done with over-expressed systems and do not reflect the in vivo scenario.

SHP-2 was coimmunoprecipitated with TrkB; this association was enhanced by MAG-Fc treatment in the CGNs (Fig. 2D), which express PIR-B, TrkB, and SHP1/2 endogenously. The association between SHP-2 and PIR-B was also enhanced by MAG-Fc treatment (Fig. 2A). We added new data to demonstrate that both SHP-2 and TrkB were coimmunoprecipitated with PIR-B following MAG-Fc treatment (Fig. 2E). The association of TrkB with PIR-B was shown in CGNs (Fig. 1H and I). We provided new data to demonstrate that PIR-B was coimmunoprecipitated with TrkB and MAG in the brains of P7 mice (Fig. 1J).

2. Is MAG's effect dependent on TrkB? What is the contribution of different receptor components such as Nogo-66 receptor, P75 and others?

In Fig. 5B and C, we provided data on whether the effect of MAG is dependent on TrkB. K252a mimicked the effect of MAG-Fc and significantly inhibited neurite growth. MAG-Fc did not further reduce neurite growth in the K252a-treated CGNs (Fig. 5B). To eliminate the possible nonspecific effect of this inhibitor, TrkB was knocked down with siRNA (Fig. 5C, lower panels), which significantly blocked neurite growth inhibition in the CGNs, and the effect of MAG-Fc disappeared (Fig. 5C). These results support that MAG reduced the basal activity of TrkB and inhibited neurite growth in the CGNs.

Concerning the different receptor complexes, we investigated the contribution of the p75 receptor to MAG-induced TrkB dephosphorylation, as p75 in complex with NgR transduces signals from MAG and Nogo-66 (Wang *et al*, 2002; Yamashita *et al*, 2002). In addition, p75 is a coreceptor that associates with Trk receptors. Indeed, we observed that p75 associated with PIR-B and TrkB when the CGNs were treated with MAG-Fc (data not shown). We explored whether p75 was also required for PIR-B/TrkB signal transduction. The CGNs from mice carrying a mutation in *p75* were employed (Lee *et al*, 1992). Trk receptors were tyrosine dephosphorylated upon MAG-Fc treatment in the CGNs from WT mice, whereas there was no change in the CGNs from mice bearing a mutation in *p75* (Fig. 4H). Thus, p75 is required for the MAG-induced tyrosine dephosphorylation of Trk receptors. Although this finding is an interesting observation, interpretation of this data is rather complex. Therefore, we think that in-depth analysis is necessary and may be the subject of another study.

3. Does endogenous PirB interact with TrkB and MAG?

The CGNs were immunoprecipitated with anti-PIR-B antibodies and immunoblotted using anti-TrkB antibodies. TrkB was detected in the immunoprecipitates obtained using anti-TrkB antibody after the cells were stimulated with 25 μg/mL MAG for 15 min (Fig. 1H). Immunoprecipitation with anti-TrkB or control antibody followed by immunoblotting with anti-PIR-B antibody also yielded similar results (Fig. 1I). Thus, PIR-B interacted with TrkB ligand dependently in the CGNs. We further assessed the interaction of these molecules by using lysates prepared from the brains of P7 mice. We added new data to show that PIR-B was coimmunoprecipitated with TrkB and MAG (Fig. 1J).

4. How about the expression of SHP1/2 in CNS neurons (different ages)? CGNs are relatively immature and other adult neurons such as retinal ganglion cells should be assessed.

We investigated the expression of SHP-1 and SHP-2 in the eyes of mice (aged P21) and added new data to Fig. 6A. Both isoforms were abundantly expressed in retinal neurons. We also confirmed their expression by western blot analysis (Fig. 6C).

5. In the experiments shown in Fig. 5, what percentages of SHP1/2 are inhibited by siRNA? How long does the silencing effect last? Both mutant mice are available and should be used to verify the conclusion. Even silencing of SHP could promotes axon regeneration, this is not sufficient to claim the contribution of PirB/TrkB/SHP. In particular, the authors just published data showing no regeneration of corticospinal axons in Pir-B knockout mice.

We determined the extent of inhibition of SHP-1/2 by siRNA. Efficient downregulation of *shp1* mRNA was found specifically in SHP-1 siRNA-transfected, but not SHP-2 siRNA-transfected, cells (Fig. 3A: 87% inhibition by SHP-1 siRNA #1; 72% inhibition by SHP-1 siRNA #2). Similarly, SHP-2 siRNA, but not SHP-1 siRNA, specifically reduced the level of *shp2* mRNA (Fig. 3B: 84% inhibition by SHP-2 siRNA #1; 69% inhibition by SHP-2 siRNA #2). Consistent results were obtained when we assessed the protein expression levels in these siRNA-transfected cells (Fig. 3C).

Concerning the duration of the silencing effect, we examined the time course of the expression of SHP-1/2 in the eyes in vivo. The lysates were prepared from eye cups at 5, 11, and 14 days after the initial injection of siRNA (Fig. 6C), and the expression of each SHP isoform was examined by western blot analysis. The results demonstrated that the silencing effect persisted for 5 to 14 days after injection of the siRNAs. Specificity of the knockdown effect of siRNAs was also confirmed (Fig. 6C).

Concerning the SHP-1 mutant mice, the lifespan of original homozygous motheaten mice (*Shp-1* is disrupted) is approximately 3 weeks (http://jaxmice.jax.org/strain/010825.html). Mice homozygous for the viable motheaten spontaneous mutation (*Ptpn6*$^{me-v}$) develop severe autoimmune disease. The lifespan of homozygous viable motheaten mice is approximately 9 weeks, with death attributed to an autoimmune pneumonitis (http://jaxmice.jax.org/strain/000811.html). SHP-2-mutant mice, a mouse model of Noonan syndrome, exhibit a gain-of-function mutation (Nat. Med., 10, 849–857, 2004). The loss-of-function mutation of SHP-2 is embryonically lethal (EMBO J, 16, 2352–2364, 1997). Therefore, we believe that the SHP mutant mice were not appropriate for our experiments. However, we agree with the reviewer that we should strengthen the data from experiments that employed siRNAs. We performed appropriate control experiments as described in Nature Cell Biology (5, 489–490, 2003). The control experiments included multiple siRNAs for the same target (Fig. 3A–C; Fig. 7), assessment of the possible off-target effects (Fig. 3A–C), and rescue by the expression of target sequences refractory to siRNA (Fig. 3D and E). Specific knockdown of target protein by siRNA in vivo was also confirmed (Fig. 6C). Thus, our data obtained by the use of siRNAs are reliable.

Finally, we employed PIR-B$^{-/-}$ mice to determine the role of PIR-B in axon regeneration. In PIR-B$^{-/-}$ mice, the number of regenerating axons was comparable to that observed in WT mice (Fig. 7E and F). Because downregulation of SHP by itself enhanced TrkB phosphorylation in retinal cells (Fig. 6D and E), we reasoned that TrkB activation as well as PIR-B inhibition may be necessary for axonal regeneration. Indeed, the level of TrkB phosphorylation was not enhanced in the retinal cells from PIR-B$^{-/-}$ mice (Fig. 6F). To test this hypothesis, we injected BDNF into the eyes of PIR-B$^{-/-}$ mice. Interestingly, BDNF injection increased axonal regeneration in PIR-B$^{-/-}$ mice (Fig. 7E and F) but not in WT mice (data not shown). These results suggest that the inhibition of PIR-B and activation of Trks is necessary for axonal regeneration (page 11, last paragraph ~ page 12, first paragraph).

Reviewer #2

1. Figure 5. The regeneration experiment shows only that SHP1 and 2 are required for regeneration of the injured optic nerve. While this is a novel finding, it says nothing about whether PIR-B or TrkB are involved in the SHP siRNA effects. Cytokines that also use SHPs regulate survival and regeneration. Therefore, the following experiments should be performed:

A. The authors have the PIR-B knockouts in hand, and an effective way to deliver siRNA to the injured optic nerve. They should show that PIR-B downregulation or knockout results in enhanced regeneration of the optic nerve, as they have shown for SHP-1/2 knockdown.

We employed PIR-B$^{-/-}$ mice to determine the role of PIR-B in axon regeneration. In PIR-B$^{-/-}$ mice, the number of regenerating axons was comparable to that observed in WT mice (Fig. 7E and F). Because downregulation of SHP by itself enhanced TrkB phosphorylation (Fig. 6D and E), we reasoned that TrkB activation as well as PIR-B inhibition may be necessary for axonal regeneration. Indeed, the level of TrkB phosphorylation was not enhanced in the retinal cells from PIR-B$^{-/-}$ mice (Fig. 6F). To test this hypothesis, we injected BDNF into the eyes of PIR-B$^{-/-}$ mice. Interestingly, BDNF injection increased axonal regeneration in PIR-B$^{-/-}$ mice (Fig. 7E and F) but not in WT mice (data not shown). These results suggest that the inhibition of PIR-B and activation of Trks is necessary for axonal regeneration (page 11, last paragraph ~ page 12, first paragraph).

B. They should perform immunoflourescence with anti-phospho-TrkB to show that SHP and PIR-B knockdown enhances TrkB activity.

We tested whether SHPs regulated the phosphorylation of Trk receptors in the dissociated retinal cells. TrkB was immunoprecipitated with anti-TrkB antibodies, and the phosphorylation level of TrkB was determined. Knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) abolished the TrkB dephosphorylation induced by MAG in retinal cells. In addition, knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) by itself enhanced TrkB phosphorylation. This could be observed after the in vivo siRNA injection into the optic nerve, as downregulation of SHP1 or SHP-2 by itself enhanced TrkB phosphorylation in the retinal neurons (Fig. 6D and E). Concerning the PIR-B

knockout, the phosphorylation level of TrkB was not significantly different between WT CGNs and PIR-B knockout (KO) CGNs (Fig. 4G). The level of TrkB phosphorylation was not enhanced in the retinal cells from PIR-B$^{-/-}$ mice (Fig. 6F). Because addition of BDNF in the PIR-B KO mice was necessary to induce axonal regeneration after optic nerve injury (Fig. 7E and F), we consider that PIR-B KO does not modulate the phosphorylation level of TrkB. We performed immunofluorescence with anti-phospho-TrkB antibodies and generated the data shown in Fig. 1A for referee-only. Although we observed phosphorylated TrkB signals in the retinas injected with SHP-1 siRNA or SHP-2 siRNA, we observed little signals in the retinas from WT or PIR-B KO mice. We considered inclusion of the data in the manuscript but decided not to include them because the immunofluorescence of the phosphorylated TrkB was not very clear. Accordingly, we provided western blots data as explained above.

Minor comments:
3. Figure 1G. Which of the two bands is PIR-B? It is running as a tight doublet in the IP, and not in the lysate blot.
We replaced the figure (Fig. 1I in the revised manuscript) to avoid confusion and added an arrow to indicate the PIR-B band.

4. Fig 2A, 2D. Both SHP-2 and PIR-B levels increase in the plus MAG lane, so I was not convinced that MAG addition increased PIR-B associations with SHP-2. This blot should be quantified. In 2D, the SHP2 band in the minus MAG/plus TrkB lane is uneven, and I am not convinced that SHP2-TrkB associations are increasing with MAG addition.
We replaced the blots with the appropriate ones (Fig. 2A and D) and provided the quantification data (graphs in Fig. 2A and D).

5. Fig. 3B, C, D and 4A. A general issue with the data in these figures is that SHP-1 siRNA or inhibitor decreases TrkB tyrosine phosphorylation and neurite outgrowth in the absence of MAG. Shouldn't it increase TrkB tyrosine phosphorylation if the authors' hypothesis is correct?
We appreciate the reviewer's important comment. To address this point, we employed retinal neurons. We tested whether SHPs regulated the phosphorylation of Trk receptors in the dissociated retinal neurons. TrkB was immunoprecipitated with anti-TrkB antibodies, and the phosphorylation level of TrkB was determined. Knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) abolished the TrkB dephosphorylation induced by MAG in retinal neurons. In addition, knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) by itself enhanced TrkB phosphorylation. This could be observed after the in vivo siRNA injection into the optic nerve, as downregulation of SHP1 or SHP-2 by itself enhanced TrkB phosphorylation in the retinal neurons (Fig. 6D and E). Therefore, the results obtained by employing the retinal neurons are consistent with our hypothesis.

6. Fig. 4A or 4D. It should be shown that either SHP siRNA or the SHP/TrkB association blocking peptide increase TrkB phosphorylation.
We generated new data by employing mouse retinal neurons. Knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) by itself enhanced TrkB phosphorylation in retinal neurons. In CGNs, the results demonstrated that knockdown of either SHP-1 (Fig. 4E) or SHP-2 (Fig. 4F) by itself did not enhance TrkB phosphorylation (Fig. 4C and D). These results suggest that SHP-1/2 suppresses the basal level of TrkB phosphorylation in the retinal neurons, but not in the CGNs.

Reviewer #3
1. In Fig 1F, I don't see a convincing difference in the TrkB blot after MAG-Fc treatment. For review, please show a series of biological replicates of this data that make a convincing statement of PirB-TrkB association promoted by MAG treatment, or else revise the point in the results/discussion.
We provided a series of biological replicates of the data (Fig. 1B for referee-only) and added the quantification data to Fig. 1H in the revised manuscript.

2. In Fig 2C, the increase in SHP2 pulled down by TrkB does not appear significant given the increase in TrkB pulled down in the IP. For review, please show a series of biological replicates of this data that make a convincing statement of SHP2-TrkB association promoted by MAG treatment, or else revise the point in the results/discussion.

We provided a series of biological replicates of the data (Fig. 1C for referee-only) and added the quantification data to Fig. 2C.

3. The results section (page 6) refers to SHP2 being pulled down into a TrkB-PirB complex as shown in Figs. 2C-D, but these data only show SHP2-TrkB association, not "complex" formation with PirB. The wording of the results (and throughout) should be revised to reflect the data.

We revised the text according to the reviewer's comment (ex; page 6, second paragraph).

4. The loss of neurite growth down to 40 um average in Fig. 4B-C may not reflect the specific importance of TrkB inhibition in MAG-induced neurite inhibition, but rather the general importance of TrkB to neurite growth, below which nothing can inhibit further (e.g. if the 40 um represents a sprouting or adhesion fundamentally different from neurite elongation). This point should be discussed in the results and discussion as an alternative interpretation of those data.

We emphasized this point in the discussion as the reviewer suggested (page 13, last paragraph).

5. In Figs. 4F-G, SHP-Trk peptide appears to inhibit the basal levels of neurite growth. Can the authors explain how that fits their model?

We found no statistically significant changes in the levels of neurite growth after treatment with the SHP-Trk peptide (Fig. 5F and G in the present manuscript).

6. What is the effect of PirB knockout on optic nerve regeneration? These experiments should be performed and included in the paper.

As the reviewer suggested, we employed PIR-B$^{-/-}$ mice to determine the role of PIR-B in axon regeneration. In PIR-B$^{-/-}$ mice, the number of regenerating axons was comparable to that observed in WT mice (Fig. 7E and F). Because downregulation of SHP-1 or SHP-2 by itself enhanced TrkB phosphorylation (Fig. 6D and E), we reasoned that TrkB activation as well as PIR-B inhibition may be necessary for axonal regeneration. Indeed, the level of TrkB phosphorylation was not enhanced in the retinal cells from PIR-B$^{-/-}$ mice (Fig. 6F). To test this hypothesis, we injected BDNF into the eyes of PIR-B$^{-/-}$ mice. Interestingly, BDNF injection increased axonal regeneration in PIR-B$^{-/-}$ mice (Fig. 7E and F) but not in WT mice (data not shown).

7. What is the percent transfection of the CGNs? Of the retinal cells in vivo? Can this be assessed by immunostaining?

Nucleofection of the siRNAs into CGNs yielded almost 100% transfection efficiency (page 7, first paragraph). For the retinal cells in vivo, efficient transfection of Alexa488-labeled siRNA in retinas was observed (Fig. 6B). The silencing effect determined by western blotting persisted for 5 to 14 days (Fig. 6C).

8. The number of axons regenerating in the optic nerve is exceedingly small, even if statistically significant. This should be discussed in the results and discussion section.

We discussed the issue in the results (page 11, second paragraph) and discussion sections (page 15, second paragraph).

9. Supplemental Fig 1A and B can probably be squeezed into the regular manuscript, avoiding the need to use supplemental data.

We combined these figures (Fig. 1C and D) in the revised manuscript as the reviewer suggested.

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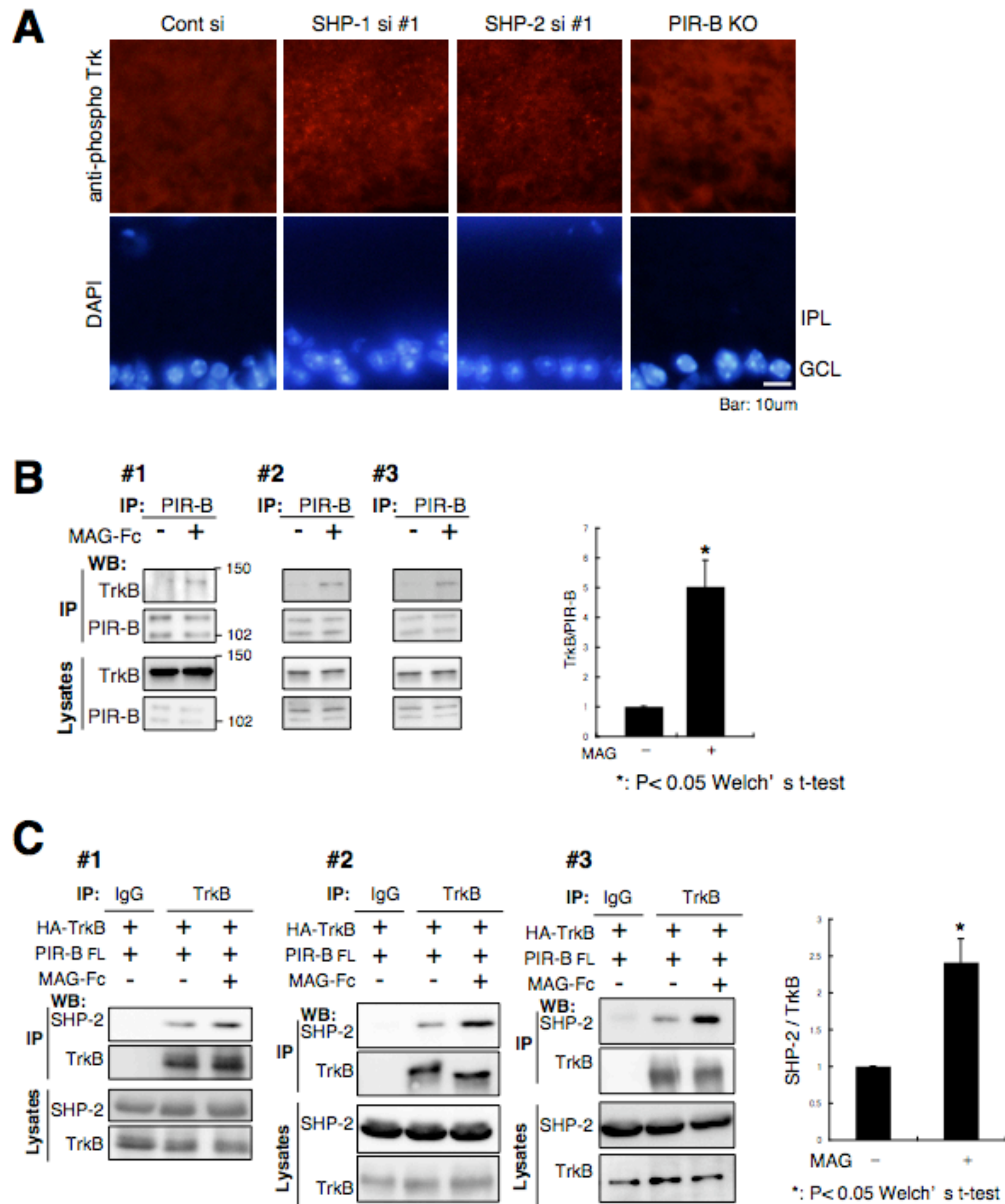
Figure 1 for referee-only



2nd Editorial Decision 01 February 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #2 and 3 to review the revised manuscript and I have now received their comments.

As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the paper. Before doing so, there are a few minor changes needed. Referee #2 suggests an alternative title and referee #3 would like you to include the BDNF data into figure 7, which I find a good idea. I would therefore like to ask you to address these last issues in a final revision. Once we receive the revision we will accept the paper. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The authors have addressed all of my concerns, and in particular, added in vivo data with the PIR-B knockout that significantly increases the impact of the findings.
My one issue is that while the title is appropriate for the non-specialist, it is too vague for those in the axon regeneration or neurotropic factor fields.
I suggest:
Myelin suppresses axon regeneration by PIR-B/SHP-mediated inhibition of Trk activity.

Referee #3 (Remarks to the Author):

Excellent revision. The authors should include the data in figure 7 on WT+BDNF that they refer to as "data not shown."

2nd Revision - authors' response 02 February 2011

Along the lines suggested by the reviewers, we revised the manuscript carefully. We revised the title of the manuscript according to the suggestion raised by referee 2. We included the BDNF data into Figure 7 as the referee 3 suggested. This has addressed the concerns raised by the reviewers.