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Limited availability of ZBP1 restricts axonal mRNA localization and nerve regeneration capacity

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Transaction Report:

(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 18 June 2011

Thank you for submitting your manuscript to the EMBO Journal. Two referees have now seen your study and their comments are provided below.

As you can see both referees find the analysis interesting, comprehensive and well done. They raise relative minor concerns with the manuscript, mostly concerning clarification of the text and data. I don't suspect that it should involve too much additional work to address the raised concerns. I would therefore like to invite you to submit a suitably revised manuscript that takes the raised concerns into consideration.

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, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for submitting your interesting study to the EMBO Journal.

Best

Editor
The EMBO Journal

REFEREE REPORTS

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Referee #1 (Remarks to the Author):

This study from the Twiss and Bassell laboratories is an important verification of the contribution of axonal RNA localization and local translation to axon growth and regeneration - indeed to the best of my knowledge it is the first study to use mouse transgenesis to definitively address this issue in vivo. The main conclusion that an RNA-binding protein, ZBP-1, is a limiting factor for RNA localization and for axon regeneration, is important new mechanistic insight on this topic. The work is comprehensive, using a diverse array of both loss and gain of function approaches, and the data is for the most part convincing and well controlled. There are however a host of minor issues, mainly arising from what appears to be overly-rushed writing, that need to be addressed before the manuscript can be published in EMBO J. Specifically:

Abstract - Please re-word the closing sentence in more succinct and clear language, e.g. "These data support a direct role for ZBP1 in transport and translation of mRNA cargos required for axonal regeneration".

Page 4, lines 10-11 - "Moreover, the how any..." - this sentence obviously needs re-wording.

On page 6, the opening sentence of paragraph 2 ("To determine if GFP plus...") is very convoluted, please re-phrase for clarity.

Figure 2A: Is there a difference in soma size between AV-GFP-3'-gamma-actin neurons and the others, or is this picture not representative? If there is a soma size difference, please describe and quantify.

Figure 2B: axon length and growth cone area are stated to be in mm units, I assume the correct range should be micro-m? Also I would suggest to move this panel, which is actually a table, to supplementary material as a table, and use the space to show representative images for the quantified data of Figure 2C.

Fig 3A: the figure legend states that $p > 0.05$ at all distances. Did the authors mean $p < 0.05$? Also, please consider showing selected images for each panel in Figure 3, alongside the graphs.

Fig 4B: The p values are presented in a confusing manner. Generally speaking, throughout the manuscript, it would be easier for the reader if statistical significance was indicated in the figures by a code of *, **, *** for different p value ranges. Precise numbers can be provided in the figure legends if the authors or editor find this necessary.

Fig. 5B: This is a table, not a figure panel. I would suggest to move this to a supplementary table, and replace in the figure with a heat map, such as commonly used to show e.g. differential gene expression data from microarrays.

Fig 6E: Representative images from locations showing significant differences would be helpful here.

Fig 7A: Here again, a heat map would be better, with the full table as supplementary.

Fig 7C: This is an important panel, but is very difficult to understand at first glance. A heat map would be a much clearer way of representing these data.

Finally, there are a number of minor errors and lacunae in spelling and grammar throughout the manuscript. Please do a thorough check using automated spelling/grammar checking software.

Referee #2 (Remarks to the Author):

This manuscript by Donnelly et al describes the role of RNA binding proteins to drive axonal mRNA localization and axon growth. These results reveal a role for ZBP1, a specific RNA binding protein, in determining the amount of β-Actin and GAP-43 mRNA in axons, that in turn contributes

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to axon growth both in vitro and in vivo. First the authors show that exogenous 3'UTR of β-actin, but not 3'UTR of gamma actin decreases axonal levels of endogenous β-actin mRNA, leading to a decrease in axonal outgrowth in cultured adult sensory neurons. Then they show that 3'UTR of β-actin transgene expression decreases axon outgrowth of DRG neurons in culture and nerve regeneration in vivo. These growth deficits are related to limited levels of ZBP1, since ZBP1 transfection reverses the growth deficit in the β-actin transgene and also increases axonal outgrowth in wt DRG neurons. They further show that ZBP1 is needed for axonal localization of Gap-43, and that ZBP1+/- mice have limited regenerative capacity and deficit for β-Actin and GAP-43 mRNA transport. This study is very interesting and is likely to be highly significant for understanding the mechanisms that control RNA localizations in axons and maybe suggest new strategies to improve axon regeneration. The authors present an elegant set of data, which is generally convincing, but there are a number of concerns that need further clarifications.

1. The images of cultured DRG showed in Figure 2A appear totally different from the one showed in Fig 1A, even if the growth condition and the markers used are the same according to the figure legends. DRG in Figure 2A display a more elaborated branching and growth pattern compared to Figure 1A. Quantification of axon branching and number of processes showed in 2B was done manually according to the method section, but based on images provides in Figure 2A, it is unclear how manual scoring was possible, especially for the branching. The authors thus need to explain the differences in morphology between the cultures in Figure 1A and 2A, and to find a more convincing way to measure axon branching and number of processes (i.e. Sholl analysis with Image J).
2. In Figure 5C, to further relate the increase in actin and GAP43 amount in axons with ZBP1 capacity of mRNA transport and release and with the regenerative ability, the authors should perform the experiments using also the 2 mutated forms of ZBP1, as done in Figure 4B. The y axis of Figure 5C should also be more precisely labeled, to differentiate total level form axonal level of endogenous mRNA
3. Figure 5D reports just one western blot band. It is thus not clear if it belongs to brain or spinal cord extracts as stated in the legend. If the experiment was done in both, then both western blots should be shown. Also it is not clear if the blot shown belongs to an IP performed with the β-actin or γ-actin extract. The efficiency of ZBP1 IP need to be the same with both extract for the quantification to be meaningful and any comparison made. The authors should confirm that the ZBP1 levels are equal in both transgenic lines and that ZBP1 IP efficiency is similar in both extracts.
4. Since the authors underline that a critical advance in their study is that the levels of ZBP1 can limit regenerative capacity, it is important to demonstrate that the protein levels of ZBP1 is increased by the conditioning injury in adult DRG. Showing that the conditioning injury increases ZBP1 level in DRG is also important, given that the experiments using beta and gamma actin 3'UTR point to a role of limiting amount of ZBP1 in adult DRG, but yet there is extremely low or no expression of ZBP1 in adult DRG according to Figure 6A and 6B. Also, the legend of Figure 6B does not match the Figure: "representative immunoblots from DRG and spinal cord", but only one immunoblot is shown. It would also be interesting to compare ZBP1 levels in central and peripheral branches of DRG neurons, with or without injury. If higher levels of ZBP1 in peripheral branches than in central branches are found, then the data could be related to the different regenerative capacity between central and peripheral DRG branches.
5. The quantifications are overall convincing. However, the authors should provide representative pictures for growth cone area (Figure 2B), for the in vivo experiments on sciatic nerve (Figure 3A and 3B) and for experiments on ZBP1+/- mice (Figure 6C, 6D and 6E and Figure S3B).

Minor concerns
1. Related to point 4 above, the authors should describe in more details the experimental methods for the DRG culture procedure, specifying if all of them are from conditioned injured animals. The method section refers to Twiss et al. 2000, which indeed used conditioned injured DRG.
2. More details about the transgenic lines are needed, especially when the text says (on page 11) that sciatic nerve crush activated the Tα1 promoter. The lines are only briefly described on page 6.
3. The Y axis of Fig 2B says "Number of PGC9.5+ axon profiles", but in the legend and text it appears that the marker used is neurofilament.
4. Is not clear in Figures 3A and 3B if the immunohistochemistry was done in longitudinal or cross sections.
5. Both in the text and in the legend of Figure 3A, it is indicated that there is a significant loss of GAP-43 positive regenerating axons between transgenic β-Actin mice and transgenic γ-Actin mice,

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but the legend reports p{greater than or equal to}0.05.
6. In order to relate quantifications done in Figure 4B for ZBP1 expression with quantification done in Fig 2B and 2C, the authors should also provide the quantification of absolute axon length in Figure 4B.
7. The legend of Figure 4C does not report the DRGs DIV.
8. The authors say on page 12 that DRGs of ZBP1+/- mice have only 40% ZBP1 mRNA levels of wt, but is not clear if this is a previously reported data (Hansen at al, 2004) or a a data not shown.
9. The FISH signal was quantified as (pixels/µm2) (page 23). It would be more correct to define it as number of pixel above threshhold or above noise level per µm2, since the number of pixel per surface area has to remain the same given that images are taken with the same camera at the same magnification.
10. Figure 2B reports length in mm instead of µm.

1st Revision - authors' response 19 August 2011

EMBOJ-2011-78368
Response to Previous Review

We sincerely appreciate the reviewer's careful critiques of our manuscript. Both reviewers noted that our study provides an important mechanistic advance for the field. We have made several changes to the manuscript to specifically address the each of the reviewer's comments. We hope that the reviewers and editor will agree that the changes detailed below strengthen the manuscript.

Referee #1:

1. *Abstract - Please re-word the closing sentence in more succinct and clear language, e.g. "These data support a direct role for ZBP1 in transport and translation of mRNA cargos required for axonal regeneration".*

Changed per reviewer's suggestion.

2. *Page 4, lines 10-11 - "Moreover, the how any..." - this sentence obviously needs re-wording.*

We have changed this sentence.

3. *On page 6, the opening sentence of paragraph 2 ("To determine if GFP plus...") is very convoluted, please re-phrase for clarity.*

We have changed this sentence and added further details on the transgenic mice (also requested by reviewer # 2). Note that a separate manuscript detailing these mice is now in press and referenced in the manuscript (Willis et al., Axonal localization of transgene mRNA in mature PNS and CNS neurons, J Neurosci in press).

4. *Figure 2A: Is there a difference in soma size between AV-GFP-3'-gamma-actin neurons and the others, or is this picture not representative? If there is a soma size difference, please describe and quantify.*

There is no difference in soma size with transduction by the AV-GFP-3'γ-actin and AV-GFP-3'β-actin preparations. The three subpopulations of neurons in the DRG have different soma sizes and the previous images unfortunately displayed these differences. We have replaced these images for Fig 2A.

5. *Figure 2B: axon length and growth cone area are stated to be in mm units, I assume the correct range should be micro-m? Also I would suggest to move this panel, which is actually a table, to supplementary material as a table, and use the space to show representative images for the quantified data of Figure 2C.*

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This should be μm as the reviewer pointed out. We have corrected this and moved this table to supplemental materials editing Figure 2C to add representative images for neurons from the transgenic animals as suggested by the reviewer. It should be noted that naïve neurons were used for the AV transduction and plasmid transfections while injury-conditioned neurons were used for the transgenic mice shown in Fig 2. We have detailed the differences for DIV for the naïve vs. injury-conditioned culture conditions (≥ 3 vs. 1, respectively) in the revised manuscript.

We have also included representative images of growth cones from the AV transduced cultures.

6. *Fig 3A: the figure legend states that p > 0.05 at all distances. Did the authors mean p < 0.05? Also, please consider showing selected images for each panel in Figure 3, alongside the graphs.*

Yes, that has been corrected to $p < 0.05$. We have also included representative images in the revised figure.

7. *Fig 4B: The p values are presented in a confusing manner. Generally speaking, throughout the manuscript, it would be easier for the reader if statistical significance was indicated in the figures by a code of *, **, *** for different p value ranges. Precise numbers can be provided in the figure legends if the authors or editor find this necessary.*

We have revised all Figures, Supplemental Figures, and Supplemental Tables to show designations for p values as symbols and restricted the descriptions to p value ranges (i.e., ≤ 0.05, 0.01 ...)

8. *Fig. 5B: This is a table, not a figure panel. I would suggest to move this to a supplementary table, and replace in the figure with a heat map, such as commonly used to show e.g. differential gene expression data from microarrays.*

We have moved the table to supplemental materials and included a heat map for these data. Fig. 5A has also been changed to a heat map and the values ± s.e.m. with significance presented as a supplemental table.

9. *Fig 6E: Representative images from locations showing significant differences would be helpful here.*

This manuscript has grown substantially with addition of representative images in earlier figures and addressing points 8, 10, and 11. We now include representative images of the longitudinal sections used to assess regeneration in the sciatic nerve as Fig. 3B. The analyses for Fig. 6E (and also for Figs. 3A, S3C) were performed exactly the same. We hope that the reviewer will allow us some leeway for not providing representative images for every analysis. With 7 very complex figures, 5 supplement figures, and 7 supplemental tables, we fear that adding more will exhaust the readers (and reviewers)!

10. *Fig 7A: Here again, a heat map would be better, with the full table as supplementary.*

We have moved the table to supplemental materials and included a heat map for these data.

11. *Fig 7C: This is an important panel, but is very difficult to understand at first glance. A heat map would be a much clearer way of representing these data.*

We have moved the table to supplemental materials and included a heat map for these data.

12. *Finally, there are a number of minor errors and lacunae in spelling and grammar throughout the manuscript. Please do a thorough check using automated spelling/grammar checking software.*

We apologize for these errors and have made thorough efforts to correct all.

Referee #2:

1. *The images of cultured DRG showed in Figure 2A appear totally different from the one showed in Fig 1A, even if the growth condition and the markers used are the same according to the figure legends. DRG in Figure 2A display a more elaborated branching and growth pattern compared to Figure 1A. Quantification of axon branching and number of processes showed in 2B was done manually according to the method section, but based on images provides in Figure 2A, it is unclear how manual scoring was possible, especially for the branching. The authors thus need to explain the differences in morphology between the cultures in Figure 1A and 2A, and to find a more convincing way to measure axon branching and number of processes (i.e. Sholl analysis with Image J).*

The images shown in Fig. 1A were cultures where we optimized for the FISH/IF analysis and display of combined IF and FISH signals. Those in Fig. 2A were cultured at a higher density and only IF was performed. As noted above in response to Reviewer 1's point 4 above, we have replaced the images in Fig. 2A and included representative images of growth cones for the AV-transduced cultures. We did attempt Sholl analyses on these cultures, but have not found this to convincingly represent the morphology of these adult DRG cultures. This may be due to the higher density for culturing adult DRGs in the absence of exogenous neurotrophins compared to embryonic DRGs that can be supported by addition of NGF. We do hope to move to automated microscopy methods, but budgetary constraints have not allowed us to purchase a high content screening microscope. We hope that the reviewer will agree to accept our manual counting methods. Please note that as indicated in the manuscript, these manual analyses were always performed in a blinded fashion.

2. *In Figure 5C, to further relate the increase in actin and GAP43 amount in axons with ZBP1 capacity of mRNA transport and release and with the regenerative ability, the authors should perform the experiments using also the 2 mutated forms of ZBP1, as done in Figure 4B. The y-axis of Figure 5C should also be more precisely labeled, to differentiate total level from axonal level of endogenous mRNA.*

Data for overexpression of the ZBP1^{Y396F} mutant in wild type neurons are included as ZBP$^{+/+}$ sample in Figure 7C. Since this mutant showed the dissociation between axonal RNA levels and axonal outgrowth (i.e., comparing Fig 7C to 4B), we focused our efforts on this mutant.

Experiments are ongoing to determine the complexes of proteins assembled onto β-actin and GAP-43 mRNAs, and we believe that the suggested studies with the ZBP1ΔKH mutant will prove much more relevant to those RNP analyses since the non-RNA binding mutant is likely competing for binding to other proteins in the transport RNP complex. We hope that the reviewer will accept the merit of the data included in the revised manuscript and let us retain ΔKH mutant for future submissions addressing protein-protein interactions in the axonal RNPs.

The y-axis for Fig. 5C has been relabeled.

3. *Figure 5D reports just one western blot band. It is thus not clear if it belongs to brain or spinal cord extracts as stated in the legend. If the experiment was done in both, then both western blots should be shown. Also it is not clear if the blot shown belongs to an IP performed with the β-actin or γ-actin extract. The efficiency of ZBP1 IP need to be the same with both extract for the quantification to be meaningful and any comparison made. The authors should confirm that the ZBP1 levels are equal in both transgenic lines and that ZBP1 IP efficiency is similar in both extracts.*

Fig. 5D shows control IP from spinal cord extracts for antibody specificity – specifically, this is the IgG control IP and anti-ZBP1 IP in lanes 1 and 2. The third lane is the input lysate showing enrichment of ZBP1 in the IP. At both RNA and protein levels, these mice show identical levels of ZBP1. This is now included as data not shown.

4. *Since the authors underline that a critical advance in their study is that the levels of ZBP1 can limit regenerative capacity, it is important to demonstrate that the protein levels of ZBP1 is increased by the conditioning injury in adult DRG. Showing that the conditioning injury increases ZBP1 level in DRG is also important, given that the experiments using beta and*

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gamma actin 3'UTR point to a role of limiting amount of ZBP1 in adult DRG, but yet there is extremely low or no expression of ZBP1 in adult DRG according to Figure 6A and 6B. Also, the legend of Figure 6B does not match the Figure: "representative immunoblots from DRG and spinal cord", but only one immunoblot is shown. It would also be interesting to compare ZBP1 levels in central and peripheral branches of DRG neurons, with or without injury. If higher levels of ZBP1 in peripheral branches than in central branches are found, then the data could be related to the different regenerative capacity between central and peripheral DRG branches.

There is only a modest increase in ZBP1 with injury conditioning and ZBP1 has not arisen as a hit from transcriptome publications for injured DRGs (e.g., Table S2 in Michaelevski et al. Sci. Signal. 3:ra53). Though timing of studies vary and ZBP1 may be upregulated at later time points after nerve injury, it seems that increase in ZBP1 does not account for the injury-conditioning effect. This was disappointing to us. Note that the current manuscript is the first to show any role for an RNA binding protein for axonal regeneration in adult animals – consequently, we believe the data as now submitted represent an advance sufficient to warrant publication in the EMBO Journal.

The legend for Fig. 6A-B now reads that a representative RT-PCR and immunoblot are shown.

We have not compared the levels of ZBP1 in in peripheral and central branches. Considering the modest increase in ZBP1 levels with peripheral injury-conditioning and competition between mRNAs for peripheral localization mentioned above, comparing peripheral and central branches with lesion is unlikely to yield insight into different regenerative capacities of these PNS and CNS axons. In a manuscript in press at J. Neurosci. and referenced in the current revision (Willis et al. Axonal localization of transgene mRNA in mature PNS and CNS neurons J Neurosci in press), we show that the GFP-3'β-actin mRNA does localize into central sensory processes after contusive spinal cord injury. We are keenly interested in the possibility that increasing ZBP1 may increase regenerative capacity in vivo, including regeneration of axons in CNS, and we have just received extramural funding to pursue this possibility. Thus, we focused the current work on PNS.

5. *The quantifications are overall convincing. However, the authors should provide representative pictures for growth cone area (Figure 2B), for the in vivo experiments on sciatic nerve (Figure 3A and 3B) and for experiments on ZBP1+/- mice (Figure 6C, 6D and 6E and Figure S3B).*

We now provide images of growth cones and neurons from transgenic mice for Fig. 2 and representative images for Figs. 3 and 6E (as supplemental Fig. S5).

Minor concerns

1. *Related to point 4 above, the authors should describe in more details the experimental methods for the DRG culture procedure, specifying if all of them are from conditioned injured animals. The method section refers to Twiss et al. 2000, which indeed used conditioned injured DRG.*

We have provided more extensive details on the culture methods. Note that we used both injury-conditioned and naive DRG cultures in the referenced paper from 2000.

2. *More details about the transgenic lines are needed, especially when the text says (on page 11) that sciatic nerve crush activated the Tα1 promoter. The lines are only briefly described on page 6.*

We have included more details on the transgenic mice used in both the Results and Materials and Methods sections. Furthermore, we have referenced a paper now in press that fully characterizes RNA localization in these mice (Willis et al., Axonal localization of transgene mRNA in mature PNS and CNS neurons, J Neurosci in press).

3. *The Y axis of Fig 2B says "Number of PGC9.5+ axon profiles", but in the legend and text it appears that the marker used is neurofilament.*

The legend now correctly reflects the PGP9.5 marker that was used for the figure. Note that we have used multiple markers for these experiments as shown in Suppl. Fig S3A.

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4. *Is not clear in Figures 3A and 3B if the immunohistochemistry was done in longitudinal or cross sections.*

Longitudinal sections were used. This is now reflected in the figure legend.

5. *Both in the text and in the legend of Figure 3A, it is indicated that there is a significant loss of GAP-43 positive regenerating axons between transgenic β-Actin mice and transgenic γ-Actin mice, but the legend reports $p \geq 0.05$.*

The p value should have read < 0.05. This editing error is now corrected.

6. *In order to relate quantifications done in Figure 4B for ZBP1 expression with quantification done in Fig 2B and 2C, the authors should also provide the quantification of absolute axon length in Figure 4B.*

We have provided absolute average axon lengths ± s.e.m. for each sample parenthetically in Fig. 4B. In reading the Reviewer's comments on these cultures, it is clear that we did not adequately detail the differences in culture conditions in the initial EMBO J submission – we have attempted to clarify culture durations and conditions fully in the Results, Materias and Methods, and Figure Legends. Note that naïve neurons were used for Fig. 4B (3 DIV) since these cultures needed time for expression of transfected constructs. Injury-conditioned neurons (1 DIV) were used for initially evaluating axonal growth from these DRGs from the transgenic mice (Fig. 2D). Consequently, these two cannot be directly compared. However, expression of GFP3'β-actin transgene mRNA clearly decrease axonal outgrowth in both conditions.

7. *The legend of Figure 4C does not report the DRGs DIV.*

DIV is now reported in the legend.

8. *The authors say on page 12 that DRGs of ZBP1+/- mice have only 40% ZBP1 mRNA levels of wt, but is not clear if this is a previously reported data (Hansen at al, 2004) or a data not shown.*

This is from our own analyses if DRGs, so data not shown. Hansen did not analyze the DRGs. Note that we provide error for this data.

9. *The FISH signal was quantified as (pixels/µm2) (page 23). It would be more correct to define it as number of pixel above threshhold or above noise level per µm2, since the number of pixel per surface area has to remain the same given that images are taken with the same camera at the same magnification.*

Thank you for catching this oversight. We have corrected this to state that the signals are above the background from negative controls (scrambled digoxigenin-labeled hybridization).

10. Figure 2B reports length in mm instead of µm.

This has been corrected.

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