MiR-134-dependent regulation of Pumilio2 is necessary for homeostatic synaptic depression

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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.)

Editor: Anne Nielsen

1st Editorial Decision 26 February 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusually long duration of the review process in this case. Your study has now been seen by two referees whose comments are shown below.

As you will see from the reports, both referees highlight the interest in describing a new regulatory pathway in homeostatic synaptic depression and would consequently support the publication of a significantly revised manuscript in The EMBO Journal. However, they do both raise a number of concerns that will have to be addressed in full for such a revised version.

In particular, referee #1 finds that further mechanistic insight on the connection between Pum2 and GluA2 is required to bring the story to the level of significance required for publication in The EMBO Journal. This concern is reflected by referee #2 who points out that the manuscript currently appears somewhat fragmented and that further insight on GluA2 regulation as well as the broader functional integration with the Plk/CDK2 pathway is required. In addition, both referees point to a number of technical issues related to data presentation, statistics and time courses that all have to be amended in a revised manuscript.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised
version of the manuscript, addressing the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version. Please feel free to contact me with any questions related to referee comments and to experimental work required for the revised manuscript.

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://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

The authors had previously shown that microRNA134 is dendritic/synaptically localized and promotes outgrowth by inhibiting translation of the mRNA encoding for the translational repressor Pumilio2 (Schratt et al., 2006; Fiore et al., EMBO J 2009). Based on these published findings the authors address here the effect of microRNA 134 on homeostatic synaptic depression in primary hippocampal neurons. They show that an initial phase of synapse elimination is then followed by a reinforcing phase of synaptic downscaling that involves downregulation of Pumilio2.

Because a functional link between Pum2 and microRNA 134 has already been established more data in support of the additional effect of microRNA 134 on synapse elimination and homeostatic depression needs to be provided. Furthermore, the authors should try to address (at least partially) the mechanism regulating the switch, mediated by the microRNA 134-Pum2 complex, between synapses formation and elimination.

Points to address
1) Ratio of excitatory/inhibitory synapses should be evaluated upon microRNA 134 silencing and overexpression.
2) Downregulation of synapses due to a toxic effect of a GABA receptor antagonist Picrotoxin has to be excluded.
3) microRNA134 targets several dendritic mRNAs, is the minor effect they observe specific for Pum2?.
4) Several dataset do not have statistical significance (i.e. Figure 2a, showing that microRNA 134 is upregulated upon 8 and 48 hrs of Ptx treatment).
5) There is no direct evidence that the endogenous Pumilio mRNA is translationally repressed during homeostatic depression.
6) Using photoactivation and fluorescence recovery after photobleaching of fluorescently tagged AMPAR the authors could definitively show that scaling down due to micro134 activity upon GABA inactivation decreases the steady-state accumulation of synaptic AMPAR.
7) A time course (8-24-48h) showing the expression of microRNA, Pumilio2 mRNA and Pum2 protein is necessary.
8) The authors claim that the peak of microRNA induction during chronic increase in network activity coincides with the onset of synaptic elimination but this does not seem to be the case (8 hrs versus 48 hrs).
9) Some of the data do not have statistical significance. I believe the authors have forgotten to add it
(i.e. Figure 2)
10) The timing of the different experiments is a bit puzzling. Sometimes the authors use 8hrs treatment, other times 24 hrs or 48hrs.

Referee #2:

Fiore et al. investigate molecular mechanisms of homeostatic plasticity using excess excitation in the presence of picrotoxin (Homeostatic Synaptic Depression; HSD), analysis of spine synapse sizes and numbers in dissociated rat hippocampal cultures, and the hypothesis that Mir134 might have an important role in this form of plasticity due to its roles in activity-dependent neuronal regulation. The two main results are: 1) HSD involves first synapse elimination and then a structural and functional downscaling of the remaining synapses; 2) identification of a pathway involving activation of Mir134 in dendritic compartments, downregulation of Pum2 and downregulation of surface GluA2 (but not GluA1) that promotes structural (but not functional) synapse loss and size reduction.

These findings are of substantial general interest to neuroscientists. The pathway is the third reported, besides one involving CaMK2/4 (in slice cultures) and one involving Plk2/CDK2/SPAR (in dissociated cultures). The dissociation of GluA1 versus GluA2 regulation is particularly interesting. Weaknesses of the study include the fact that the pathway is only defined in a fragmented way and the involvement of Pum2 in GluA2 regulation is not documented. A second weakness is that in view of previous results it would be important to provide experimental evidence addressing how this new pathway might interface with the two previous ones, particularly the Plk2/CDK2 pathway that was also reported in dissociated cultures. With the additional information and a better documentation of variation and statistical significance in the data (see below) the paper would provide the kind of mechanistic advance appropriate for publication in EMBO Journal.

Specific points:

1) The authors link Mir134 to Pum2 and synapse loss/shrinkage. They further implicate GluA2 (but not GluA1) regulation in the process. In order to complete identification of the novel regulatory pathway it would be important to address the role of Pum2 downregulation in the downregulation of surface GluA2 (does it act through GluA2?).

2) This is the third pathway identified in this kind of homeostatic plasticity. It appears to specifically regulate structural aspects of synapse loss and shrinkage and it would be important to provide some experimental evidence as to how it might intersect with one or the other pathways, in particular the Plk2/CDK2/SPAR pathway. Is this particular pathway unique in its regulation of structural aspects? Are there likely overlaps between the pathways?

3) The effects described in the paper are quite small. Ten neurons were analyzed per condition which might be a relatively small number given the size of the effects. More importantly, the authors do not provide statistical information in the main text of the manuscript. This should be fixed and information concerning the variability and robustness of the findings should be included (after all, for example, one might not necessarily expect that these in vitro cultures consist of homogeneous populations of neurons).

1st Revision - authors' response 28 May 2014

General points raised by both referees:

The referees’ comments were extremely helpful in order to significantly improve our manuscript. Specifically both reviewers asked for more mechanistic data on the pathway downstream of Pum2 with special emphasis on the mechanisms that might regulate GluA2 levels during HSD. We have
addressed this question with new experiments that are now presented in an entirely new Figure (Figure 7). We first provide evidence that Pum2 downregulation is necessary for miR-134 dependent GluA2 downscaling (Fig. 7A). We then identified the Plk2 3’UTR as a direct Pum2 target and investigated the relevance of this novel interaction for GluA2 regulation. We validated by CLIP that the Plk2 mRNA is bound by the endogenous Pum2 protein in rat hippocampal neurons (Fig 7B-D), consistent with Pum2 being a translational repressor of Plk2 expression in unstimulated neurons. Further consistent with this hypothesis, we show in Figure 7E and F that elevated Pum2 levels interfere with Plk2 activity, since Pum2 overexpression prevents Plk2-dependent SPAR degradation in Ptx treated cells. Finally, we provide functional evidence that Plk2 is downstream of miR134/Pum2 in homeostatic synaptic depression (Figure 7G), since overexpression of Plk2 reinstates downscaling of GluA2 surface receptors in the context of ectopically expressed Pum2. Taken together, we now functionally integrated the Plk2 pathway with miR134/Pum2 regulation, via a direct protein-RNA interaction between Pum2 and the Plk2 3’UTR. We further provide a rationale for the mechanism of GluA2 internalization, since known effectors of the Pum2 target Plk2 (SPAR, NSF) have well-established roles in GluA2 trafficking.

Specific points raised by individual referees:

Referee #1

1) The reviewer asks to evaluate the effect of Ptx and miR-134 inhibition on inhibitory synapses. In Supplementary figure 1C, we now show that neither Ptx nor miR-134 inhibition have any significant effect on inhibitory synapse density (discussed on page 7). We therefore believe that the major target of miR-134 during HSD are excitatory synapses.

2) The referee mentions that a toxic, non-specific effect of Ptx on synapse elimination should be excluded. We first would like to note that such potential toxic effects of Ptx on hippocampal neurons at the concentration we used for our experiments have not been previously reported in the literature (Seeburg et al., 2009; Tatavarty et al., 2013; Sun and Turrigiano, 2011). Furthermore, we provide experimental evidence that Ptx has not detrimental effect on the health of our cultures by showing in fig. S1A that there is no difference in the average number of cells and average number of apoptic nuclei between vehicle and Ptx treated cells (discussed on page 6).

3) The referee asks whether miR-134 specifically targets Pum2 in dendrites in the context of HSD. In addition to Pum2-luc, we tested other validated miR-134 targets (Limk1, Creb1) in the luciferase assay, and did not observe regulation of these reporters by Ptx (data not shown). We have now stated this more clearly in the results on page 10. In addition, we observe a downregulation of Creb1 protein by Ptx in the cell bodies (Fig. 5C, D), whereas Creb1 protein is absent from processes. However, since overexpression of Creb-VP16 has no effect on Ptx-induced synapse elimination (Fig. 6A), this Creb1 regulation is not involved in HSD and therefore also likely independent of dendritic miR-134. Taken together, our data argues that miR-134 likely targets specific mRNAs in dendrites in the
context of HSD, although we do not want to rule out that targets other than Pum2 are also involved.

4) The referee requests to provide statistical information for a few datasets, referring to Figs 2A and B. We now show that miR-134 expression is significantly induced by Ptx compared to another neuronal miRNA (miR-99b) at the 8h time point in Fig.2A. In Fig. 2B, we now indicate that the induction of miR-134 in Ptx treated neurons is statistically significant compared to vehicle treated neurons.

Since both reviewers pointed out that the information about our statistical methods was unclear we have now added a separate paragraph about statistics to the material and methods part (page 21). P-values and number of cells analyzed (N) for each experimental condition are always indicated in the respective figure legends.

5) The reviewer states that there is no direct evidence that the endogenous Pumilio mRNA is translationally repressed during HSD. We agree that the datasets provided do not directly show that Pum2 mRNA is translationally repressed by miR-134, although this is the canonical mode of regulation employed by neuronal miRNAs. To unambiguously show effects on mRNA translation, one would have to perform either ribosome profiling or polysome fractionation in ptx-treated neurons in the context of miR-134 inhibition. While this, although challenging, should be possible in principle, we want to emphasize that we selectively observed ptx regulation of Pum2 in the dendritic compartment. Therefore, the aforementioned experiments would have to be performed in a compartmentalized culture system, where starting material is notoriously limiting. Taken together, the clarification of this exciting point is in our view beyond the scope of the current manuscript revision. We are however confident that with our data from reporter assays (Fig. 5A, B), biochemical fractionation (Fig. 5C-E) and functional epistasis experiments (Fig. 6), we provide strong evidence for an essential role of miR-134-dependent Pum2 downregulation in HSD. In addition, we could show in a previous publication (Fiore et al., EMBO J. 2009) that miR-134 represses endogenous Pum2 protein production in hippocampal neurons.

6) The referee suggests that photoactivation and fluorescence recovery after photobleaching of fluorescently tagged AMPAR could be used to demonstrate an effect of miR-134 on steady-state accumulation of synaptic AMPAR. We have now attempted a similar experiment, using time-lapse imaging of the fluorescence loss after photactivation of a previously described paGFP-GluA2 (Tatavarty et al., J. Neurosci. 2013; new Fig. S6). As expected, we observed accelerated loss of surface paGFP-GluA2 from dendritic spines over a period of 50 min in ptx-treated neurons compared to vehicle treated neurons (Fig. S6, left panel). This result validated our experimental setup. Consistent with a role for miR-134 in GluA2 internalization, we observed that the ptx effect was occluded in Antimi-R134 transfected neurons (Fig. S6, right panel). Together, this experiment fully supports our conclusions drawn from results presented in Fig. 2-4.

7) The referee asks for a time course of miR-134, Pum2 mRNA and protein expression. We have now added the requested experiments in supplementary figure S2 of our revised
manuscript. In figure S2B we show that Ptx induces upregulation of Pum2 protein at the whole cell level. In figure 5C and –D we show that the upregulation persists in cell bodies at 48hrs. The time course of the Pum2 mRNA expression after Ptx treatment is shown in Fig. S2C. We refer to these findings on page 11 of the revised manuscript.

8) The referee argues that miR-134 induction does not coincide with synapse elimination at 8h Ptx treatment. We believe that this is a misperception, since in Fig. 1E and F we show that synapse elimination is in fact the earliest event of HSD and already observable after 8h of Ptx treatment (discussed on page 6). This exactly matches the peak of miR-134 induction as shown in fig.2A and B (discussed on page 7). Accordingly, we show in supplementary figure S3A (discussed on page 7) that miR-134 is necessary for homeostatic synapse elimination already after 8h of Ptx. Taken together, induction of miR-134 and synapse elimination are highly coincident.

9) See our reply to point 4 of this referee.

10) The referee is puzzled by different time points used for some experiments. We discuss on page 7 of the manuscript why we choose the 48h time point for the majority of our functional experiments: “Since we observed that the effects on HSD parameters (synapse downscaling and elimination) were most robust 48 h after the induction of chronic activity, we used 48 h Ptx treatment (DIV18-20) for all following experiments.”

We did use different time points only when it was necessary to assess the onset of synaptic elimination, synaptic downscaling and the miR-134 requirements for these aspects of HSD (fig. S1C and S3 respectively). We discuss this on pages 6-8.

Referee #2:

We are pleased that this referee states that “These findings are of substantial general interest to neuroscientists. We are confident that in our revised manuscript, we have addressed all major concerns raised by this referee.

1. The referee argues that it would be important to address the role of Pum2 downregulation in the regulation of surface GluA2. We have now performed an experiment directly addressing this point (new Fig. 7A), and found that shRNA-mediated knockdown of Pum2 reinstates downscaling of GluA2 surface receptors in the context of miR-134 inhibition. This experiment conclusively demonstrates that Pum2 downregulation is downstream of miR-134 function in HSD.

2. The referee asks for experimental evidence about a possible intersection of the miR-134/Pum2 and Plk2/CDK2/SPAR pathways. We have now addressed this point in our revised manuscript and surprisingly identified a direct interaction between the miR-134 target Pum2 and the Plk2 mRNA (Fig. 7B-D). Moreover, we found that Pum2 is involved in the regulation of SPAR, a substrate of Plk2, during HSD (Fig. 7E-F). Finally, and most importantly, we found that ectopic Plk2 expression is sufficient to reinstate downscaling of
surface GluA2 clusters in the context of Pum2 overexpression. Together, this data suggests a direct link between these pathways, whereby miR-134/Pum2 act upstream of Plk2 to control GluA2 internalization in response to chronic activation.

3. Following the suggestion of this referee, we have now improved our presentation of statistics, both in the main text and figure legends. Please refer to our reply to point 4 raised by referee #1 for further details.

2nd Editorial Decision
03 July 2014

We have now heard back from both referees concerning your revised manuscript and their reports are included below.

As you will see, while ref#2 is fully satisfied with the revised manuscript, ref#1 does raise a few technical points with the newly included data on Plk2 regulation. More specifically, this referee finds that the manuscript would be greatly strengthened by the inclusion of Western blot data for the reduction in Plk2 and SPAR levels downstream of Pum2. The referee also recommends a more careful presentation of the CLIP data to ensure specificity in the observed interaction. In light these concerns, I would therefore ask you to include this additional data - if obtainable within a reasonable timeframe - in a final revised version of the manuscript.

I would also encourage you to include the (admittedly negative) data for miR134-target interactions in HSD that you mention as 'data not shown' in your response to ref#1's point 3. We do not generally allow references to unpresented data and we would prefer to have this additional piece of information included in a supplementary figure.

In addition, I would need you to address the following, editorial points:

As of Jan 1st 2014 every paper published in The EMBO Journal includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

We now also encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

Please feel free to contact me with any questions related to the points outlined above. Thanks again for submitting your work to The EMBO Journal, I look forward to receiving the final revised version of the manuscript.

REFEREE REPORTS:

Referee #1:

In response to both reviewers who were asking for the mechanism linking Pum2 dysregulation and GluA2 clustering, the authors now present the Polo-like kinase Plk2, showing

1. It is an in vivo target of Pum2, by crosslinking/IP (CLIP) experiments;
2. Homeostatic depression, the experimental paradigm of the paper, has an effect on SPAR, a downstream target of Plk2.
3. Plk2 overexpression rescues homeostatic depression that is lost upon Pum2 overexpression.

While this kind of mechanism is what is needed to make the paper a real advance with respect to the previous paper by the same group, I feel that Plk2 has not been established as Pum2 effector:

- Most crucially, the authors do not show that Plk2 is actually dysregulated, e.g., by Western blotting.
- Even the degradation of SPAR, which would be an indirect effect of Plk2 dysregulation, is not by all means secure: the reduction of SPAR is shown only by immunofluorescence, which is always tricky to quantify, and even more so when the differences are only 10% up or down.
- Finally, also the CLIP experiment does not really show in vivo association with the mRNA: the authors did not use stringent immunoprecipitation conditions (only in the washes, the salt is moderately increased), and I am therefore convinced that a control IP without crosslinking - had it been shown - would show exactly the same signal, i.e., the immunoprecipitate contains predominantly non-crosslinked complexes/aggregates. That would also explain the presence of U6 snRNA in the immunoprecipitate which does not have a Pum2 binding site.
- Moreover, the CLIP assay is tainted by high background precipitation of Pum2 with the control IgGs as shown in panel 7C.
- There is, by the way, a typo in the M&M, section CLIP: “50 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40, 5mM NaCl” is probably meant to be “50 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40, 5mM MgCl2”

In light of these concerns, and seen also that half of my points (2., 5., 8, and 10.) were addressed by words only, the paper has not gained the strength that I think is required for a publication in the EMBO Journal.

Referee #2:

The authors have carefully revised the manuscript and have addressed all points raised in my previous review. Notably, they have now identified a target for miR-134 (Plk2) and demonstrated its relevance in regulating GluA2 surface expression.

With the new results this is a strong contribution to the field - there are not many mechanisms available for homeostatic regulation of glutamatergic synapse strength and this is a convincing case.

The paper will likely generate considerable interest among neuroscientists.

2nd Revision - authors’ response 10 July 2014

Referee 1:

1. We agree that showing by western blot that Plk2 levels are reduced upon Pum2 over-expression would provide further biochemical evidence for a direct regulation of Plk2 by Pum2. However, given that primary neurons are notoriously hard to transfect, performing such an experiment in primary neurons would require alternative efficient delivery strategies, e.g. viral delivery. Although we have successfully used rAAV-mediated delivery of shRNAs and small proteins in neurons in the past, this is not possible for large proteins like Pum2. Taken together, biochemical experiments following Pum2 overexpression would require establishing novel efficient delivery tools which is in our view beyond the scope of the present study. Nevertheless, we already provide multiple lines of evidence strongly suggesting a functional interaction between Pum2 and Plk2:

a) The endogenous Plk2 mRNA is bound by the endogenous Pum2 in primary hippocampal neurons as assessed by CLIP (Fig. 7B-D).
b) Increasing Pum2 levels interferes with downregulation of the Plk2 substrate SPAR during HSD (Fig. 7E, F), consistent with Pum2 being a negative regulator of Plk2 function.

c) Most importantly, the Pum2 gain-of-function phenotype in the context of synaptic downscaling is rescued by overexpressing Plk2 (Fig. 7G). This epistasis experiment is again consistent with Pum2 being a negative regulator of Plk2 expression and a direct demonstration of a functional interaction between these two pathways.

2. The reviewer is concerned by our choice of quantifying SPAR levels by immunofluorescence and further states that the observed effects are small (around 10%).

First, we would like to stress that, as shown in the quantification provided in Fig. 7F, the differences in SPAR signals between Pum2 overexpression and control conditions are highly reproducible between 20-30%, consistent with the effects observed upon miR-134 inhibition and in agreement with previous reports about SPAR regulation in homeostatic plasticity (e.g. Seeburg et al., Neuron, 2008).

Second, in contrast to immunocytochemistry (see references on p. 14 of the revised manuscript), Western blotting of endogenous SPAR has not been performed in previous publications in the context of synaptic downscaling.

Third, as already outlined in more detail in our response to point #1, biochemical evaluation of SPAR levels upon Pum2 overexpression would require efficient Pum2 delivery tools into primary neurons which are not available.

3. The reviewer is not convinced by the specificity of the Plk2mRNA/Pum2 interaction due to an apparent lack of stringency in the CLIP protocol.

We do not share this concern since we use the same salt concentrations and even higher detergent concentration (0.5%) in lysis and wash buffers compared to the original publication that first described a Pum2 CLIP tag in the Plk2 3’UTR in non-neuronal cells (Hafner et al., Cell, 2010). Moreover, both our binding and washing conditions are comparable to original protocols from the CLIP literature (e.g. Ule et al., Science, 2003). Together, this argues strongly against a general lack of stringency in our protocol.

The reviewer further comments that detection of the U6 RNA in the Pum2 immunoprecipitate indicates lack of specificity. We believe that this is a misinterpretation, since we actually show that U6 RNA is depleted in Pum2 compared to IgG immunoprecipitates. We presented our qPCR data as fold enrichment of the respective RNA in Pum2 versus IgG immunoprecipitates, which is common in the literature (see e.g. Karamyshev et al., Cell 2014, for a recent example). A value below 1, as is the case for U6 RNA, in fact indicates depletion in this representation.

Furthermore we show that two other transcripts, GAPDH (that is expressed at a higher level than Plk2) and LimK1, were undetectable in both immunoprecipitates. Taken together, the results from three unrelated negative controls validate the specific interaction of Pum2 with Plk2 as observed with our stringent CLIP protocol.

We have now rephrased the results part describing the CLIP experiments (p. 14 of our revised manuscript) to clarify these points.

4. Lastly, the reviewer argues that half of his/her points were addressed by words only. We disagree with this statement, since the original point #5 is actually the only one we did not address experimentally due to the extremely challenging nature of the suggested experiment (see our previous rebuttal letter). Point #2 was addressed experimentally, and points #8 and #10 only required further clarification in the text.

Referee 2:

We are happy that referee 2 finds that we have addressed satisfactorily all his/her points and thinks that our manuscript is now suitable for publication in EMBO Journal.