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miR-9-5p suppresses pro-fibrogenic transformation of fibroblasts and prevents organ fibrosis by targeting NOX4 and TGFBR2

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Transaction Report:

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Transfer Note:

Please note that this manuscript was originally submitted to The EMBO Journal where it was peer-reviewed. It was then transferred to EMBO reports with the original referees' comments and the authors' response, attached. (Please see below)

Original referees' comments and authors' response – The EMBO Journal

Referee #1:

In the present study, the authors found that overexpressed miR-9-5p exhibits an anti-fibrogenic activity, possibly through inhibiting TGFβ signaling and/or Nox4 expression. Paradoxically, miR-9-5p is induced under situations where fibrosis is enhanced. Thus, the authors discuss that miR-9-5p may be a negative feedback regulator of fibrosis induced by TGF-b. The work may be interesting as a therapeutic study. However, the biological significance of the induction of endogenous miR-9-5p remains unclear because the authors did not perform loss of function experiments. A possible role of miR-9-5p as a negative feedback molecule is thus largely speculative. In addition, although the authors convincingly demonstrated that TbRII and Nox4 are targets of miR-9-5p, it remains unclear whether miR-9-5p inhibits fibrosis by down-regulating these molecules because rescue

experiments were not conducted. Therefore, the title (miR-9-5p prevents organ fibrosis by targeting NOX4 and TGFBR2) may not be appropriate. These two major concerns should be addressed as follows:

We thank the reviewer for these suggestions. We have dedicated our efforts to address these issues.

1) To explore the biological importance of miR-9-5p induction of during fibrosis, effects of miRNA inhibitor 9 on bleomycin-induced fibrosis in a mice model as well as TGF-b signaling in cultured cells should be examined.

These effects are now shown in Fig.8 and Fig. E5. Fig. 8 shows that miR-9-5p inhibitor increased the concentration of Collagen1α1 and Fibronectin in bleomycin-treated mice. This correlated with an increased fibrotic phenotype as evidenced by histological analysis, collagen content and number of myofibroblasts. In Fig. E5 we describe changes induced by miR-9-5p inhibitor in human lung fibroblasts exposed to TGF-β and show that inhibition of mir-9-5p is associated with increased collagen, fibronectin and alpha-smooth muscle actin accumulation by using quantitative PCR, immunoblot and immunocytochemical techniques. Overall, the data from these loss-of-function experiments support the antifibrotic role of mir-9-5 p both in cells and in lung tissue.

2) Figure 1D: miR-9-5p is strikingly induced 24 h and 48 h after TGF-b stimulation. Is TbRII (TGFBR2) down-regulated at these time points? Such data can support the authors' suggestion that endogenous miR-9-5p can be a negative feedback molecule.

We thank the reviewer for the suggestion. We have preliminary data supporting that TGFBR2 is down-regulated after TGF-β treatment but the number of experiments (n=2) does not allow to formally perform statistics. We do not consider essential this information at this point but we offer for the reviewer´s sake the following graph:

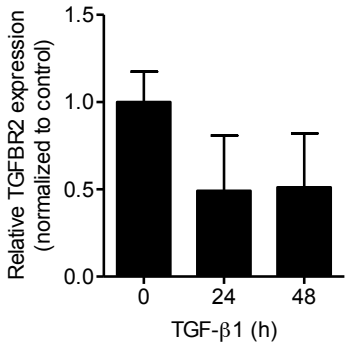

Figure: qRT-PCR analysis of TGFBR2 expression in HFL-1 cells treated with 5 ng/ml TGF-β1 for the indicated times (n = 2). Bar graphs show mean ± SEM

3) Figure 3B and C: Is re-expression of TbRII sufficient to induce aSMA and FN in pre-miR-9-5p-treated cells? If not, is re-expression both of TbRII and Nox4 required?
4) Figure 4C and D: It is not clear whether TGF-b signaling is suppressed because TbRII is down regulated. Rescue experiments using externally expressed TbRII should be performed. Alternatively, the effect of miR-9-5p on TGF-b signaling induced by the constitutively active TbRI (ALK5-TD mutant) should be examined.

As these two questions are related, we provide a common answer. Experiments were performed to “rescue” the effects of miR-9-5p, in this case its antifibrotic action, with the aim of demonstrating that the targets identified in silico and also confirmed using reporter plasmids, were in fact TGFBR2 and NOX4. Transfection with plasmids containing cDNAs for these proteins were done in the setting of previous miR-9-5p inhibition. As shown in Fig. 3E and F, transfection with these constructs resulted in a significant reduction of the miR-9-5p inhibitory effect related to the expression of α-SMA, collagen type 1 and fibronectin. Interestingly, overexpression of NOX4 had a more intense effect, an observation that might

be related to different degree of expression, given that it was not possible to use the same pCMV vector in both cases.

Minor concerns:

5) Figure 1E: The possibility that PEG-catalase affected TGF-b signaling in general is not excluded. Thus it is too early to conclude that TGF-b1-induced miR-9-5p expression was mediated by ROS. Its effect on the TGF-b-induced CAGA-Luc activity should be examined.

We thank the reviewer for his/her comment. It is true that we have not analyzed in depth the effect of PEG-catalase on TGF-β signaling. However, the aim of Fig. 1E was to prove that induction of the miRNA by TGF-β could be dependent , at least in part, on hydrogen peroxide. We believe that the fact that the PEG moiety by itself has no inhibitory effect on TGF-β induction while the incorporation of catalase to the molecule results in a powerful inhibition provides convincing evidence on this point.

6) Figure 4E: Subcellular localization of Smad2/3 is not easy to see. Images with higher magnitude would be helpful for readers.

We believe that the nuclear translocation of Smad2/3 is very clear with the magnification selected. In order to demonstrate that the effect of miR-9-5p is a general phenomenon, we wanted to show a representative picture exhibiting a great field of cells, higher magnification implies less cells per field. The differences between the cells transfected with pre-miR-NC and pre-miR-9-5p are quite clear in the pictures. In the control cells Smad2/3 presence is associated with a nuclear staining after TGF-β1 treatment. Conversely, when pre-miR-9-5p is over-expressed, the great majority of the cells present a cytoplasmic staining for Smad2/3.

7) Discussion: In the last paragraph, the authors mentioned the possibility of using miR-9-5p as a therapeutic tool for human organ fibrosis (p14). However, they also described that miR-9-5p behaves like an oncogene in the first paragraph (p10). Is there a good way to avoid this unfavorable side effect?

We have no direct information on this possibility. However, for the sake of clarity and after the recommendation of reviewer # 3 to shorten and streamline the discussion we have left out this paragraph.

8) Discussion: The role of miR21 in fibrosis should be discussed as a regulator of TGF-b signaling (down-regulation of Smad7 that inhibits TGF-b signaling).

We thank the reviewer for the suggestion. We have expanded the information on the role of miR-21 in fibrosis in the Discussion section.

Referee #2:

In their manuscript entitled "miR-9-5p prevents organ fibrosis by targeting NOX4 and TGFBR2" by Fierro-Fernandez et al describe the role of miR-9-5p during organ fibrosis. Data from the authors demonstrate that miR-9-5p expression is increased in human lung fibroblast exposed to ROS or treated with TGFb. Interestingly, they found that miR-9-5p directly targets TGBR2 and NOX1, thus regulating TGF-signaling. This data suggest that TGF-b induces the expression of miR-9-5p to regulate its own homeostatic response. Most importantly, the authors shown that the expression of miR-9-5p in lungs is increased in bleomycin treated mice and treatment mice with miR-9-5p mimics prevent experimental fibrosis. Overall, this is a very interesting and elegant study. However, additional experiments should be performed to support author conclusions.

We thank the reviewer for his/her positive comments.

Specific points:

1) The authors suggest that miR-9-5p fine-tune TGFb signaling by targeting TGFBR2 and NOX4 expression. They measured the expression of miR-9-5p pri-miRNA and suggest that the regulation of miR-9-5p levels is regulated at transcriptional levels. However, the time course experiments measuring miR-9-5p and pri-miR-9-5p expression do not suggest such regulation. miR-9-5p levels increase significantly at 24 h. Similarly, the pri-miRNA also increase at this time point (only the pri-miR-9-2 isoforms appears to be regulates at earlier time points but the expression of miR-9-2 is not regulated by TGF-b as the authors claim). The authors should measure the expression of pri-miR-9-1,2 and 3 and pre-miR-9-1,2,3 between 12 and 24 h after TGFb stimulation. Analysis of pri-miR, pre-miR and mature miRs by Northern blot should strengthen author's conclusions.

We certainly appreciate and understand the caveat posed by the reviewer. It is true that the experiments hereby suggested would certainly prove the assumption of transcriptional regulation of miR-9-5p but we feel these experiments should form part of a global study dedicated to this aspect where transcriptional regulatory elements for miR-9-5p should also be identified. Regarding the lack of close temporal correlation between pri-miRNA and miRNA levels, it is possible that different cellular sensitivity to TGF-β along several experiments could account for this observation. However, please note that Fig. 1D shows a huge change in miR-9-5p expression between 8 and 24 hours whereas pri-miR-9-1 isoform, which is sensitive to TGF-β, is already up-regulated at 12 h (Fig. E1, panel B), thus suggesting the expression of this isoform precedes that one of the mature miRNA.

2) Bioinformatic analysis of SMAD binding sites in miR-9-1 and miR-9-3 promoters is not sufficient to demonstrate TGFB transcriptional regulation of both miRNAs. The authors should perform promoter studies by cloning the miR-9-1 and miR-9-3 promoters in reporter constructs. Additional CHIP experiments using SMAD Ab should directly demonstrate the transcriptional regulation of miR-9-1 and miR-9-2 expression by TGFb-SMAD signaling pathway.

Again, we believe that the suggestion of the reviewer is full of sense and insight. As we state above we think these approaches should be part of an in-depth study regarding the transcriptional regulation of miR-9-5 p by TGF-β.

3) The authors nicely shown that miR-9-5p overexpression reduces fibrosis in mice. However, these set of experiments do not directly assess the TGF-b-miR-9-5p-TGFBR2 regulatory loop. It would be important to demonstrate that antagonism miR-9 increases lung fibrosis in mice treated with bleomycin.

We agree with the reviewer and in line of the response to Reviewer #1, we beg him/her to refer to the answer provided in point 1 to the aforementioned reviewer.

Referee #3:

Fierro-Fernandez and colleagues report the anti-fibrogenic actions of miR-9-5p. They focus mainly on diseases such as IPF and work with cultured fibroblasts, bleomycin model of pulmonary fibrosis in mice, and peritoneal derived mesothelial cells. They focus mainly on targets NOX4 and TGFBR2 as feeding into the known fibrogenic pathways important in IPF. Most of the in vitro data are technically sound. The in vivo work, however, needs improvement. It would benefit from increased mice in each comparative cohort to make their in vivo findings believable. Furthermore, the lentivirus delivery process needs to be better characterized to be believable (see below). More importantly, a number of necessary experiments are missing to make the conclusions of this paper believable (as detailed below). Furthermore, I have questions regarding the novelty of the work-I think this may be better served in a specialty journal rather than general interest.

We thank the reviewer for the positive statements and constructive criticisms offered in the introductory paragraph.

Major Comments:

1. Novelty: My enthusiasm for this paper is dampened as the elucidated pathway is only novel in that miR-9-5p is now one of several microRNAs known to be relevant in fibrosis. The proposed downstream targets of this miRNA are already known important players in fibrosis and really do not advance our understanding of fibrotic disease. I suppose one could argue that the in vivo delivery of miR-9-5p could have therapeutic potential in IPF, but much more data would have to be generated for that to be a convincing conclusion.

We understand the doubts of the reviewer although we do not share them to a full extent. We believe this is the first report to present miR-9-5p as both a redoximiR and a fibromiR. While it is true that several miRNAs have been reported in the context of fibrosis there are elements of singularity in this study concerning its participation in two different settings (IPF and peritoneal fibrosis) and the pattern of response elicited by TGF-β. In this sense there are not so many examples whereby this profibrotic cytokine promotes signals destined to self-limit its harmful effects. Beyond these arguments, new data are now provided (see below), which we think may contribute to mellow the perception of the reviewer.

2. Conclusion that miR-9-5p is a homeostatic brake to fibrosis: The utility of endogenous miR-9-5p upregulation as a homeostatic brake is not entirely clear, making the significance of this finding a bit vague. More importantly, the authors leave out multiple key experiments to show that miR-9-5p is necessary as such a brake. To do so, they will need to perform in vitro and in vivo inhibition experiments using anti-miR-9-5p strategies in IPF (or use a miR-9-5p KO mouse). In this era of biology, such experiments are standard in proof of microRNA function and the lack of such experiments are concerning that this biology is in fact not robust.

We thank the reviewer for this comment. Data addressing this major concern have now been included in Figs. 8 and E5. Please see response to point 1 of reviewer #1.

3. Most mouse experiments only use N=4 mice per group. While the statistics show significance, we know that at least for a majority of the in vivo mouse experiments, there should be least N = 7-8 mice per group to allay the concerns of biologic variability.

We agree with the reviewer on the fact that the higher the number of mice the more robust the conclusions drawn become. However, the n=4 is the minimum number used and we have never excluded potential outliers or use tests that could favor our interpretations. Hence, we feel reasonably comfortable with this approach. In the new experiments performed we have used more animals per group, having in mind the considerations of the reviewer.

4. The authors focus on targets NOX4 and TGFBR2 as important for miR-9-5p in these antifibrotic effects. However, the methods to get there and experimental proof of this need improvement. First, a great many microRNAs tend to target TGFb-superfamily signaling molecules as targets. Thus, the fact that miR-9-5p also does brings up a major concern that it is only one of many that are necessary as a coordinated group to affect TGFb-specific actions. The authors do not show any data that these targets are important in miR-9-5p specific effects (i.e., forced expression of NOX4/TGFBR2 in the presence of miR-9-5p to reverse the microRNA actions) - these would be important both in vitro and in vivo.

We agree with reviewer on this important point. Experiments have been done to address it and for details we beg the reviewer to refer to the answer provided to reviewer #1 in point 4.

5. Lentiviral miR delivery: Fig. E8 needs to be substantially improved to convince the reader of where lentiviral miR-9-5p is being expressed and to what degree. First, as expected, the frozen sections of lung show substantial autoflourescence which makes interpreting an increase of GFP signal very difficult. Another color likely needs to be used or perhaps specific IHC staining for lentiviral products needs to be performed. Furthermore, we don't know in what cell types this miRNA is being expressed after lenti delivery. It should be confirmed in fibroblasts specifically rather than just in lung tissue in general.

We agree with the reviewer regarding the green autofluorescence of the mice lungs. For this reason we selected a control mice lung without lentiviral administration and pictures with the same image settings as mice lung to which lentivirus had been administrated were taken. This precaution allows us to assume that all the green fluorescence observed corresponds to cells infected with the lentivirus. However it would be appropriate to use a lentivirus with other markers like RFP o YFP. With the orotracheal instillation the delivery of the lentivirus is systemic and the reviewer is also right indicating that we cannot conclude in which cells miR-9-5p is expressed. This issue should be addressed in the future.

6. Does pre-miR-9-5p allow for the same level of expression of this miRNA as endogenous situations of TGF-specific induction or ROS induction?

We thank the reviewer for this insightful comment. We think that the level of expression of endogenous miR-9-5p under specific stimuli (Fig.1 C,D) is certainly lower than that attained with pre-miR transfection (Fig. E4) or lentiviral strategies (Fig. E9B) and this may account for the incomplete antifibrotic effect observed, as mentioned in the discussion.

7. Discussion seems too long and should be streamlined.

The discussion has been shortened and hopefully streamlined.

1st Editorial Decision – EMBO reports 11 June 2015

Thank you for the transfer and the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from the referees that were asked to assess it.

As you will see, while both referees acknowledge that the study has been improved, they still raise concerns. After discussing these concerns with my colleague here, we have decided that the first concern by referee 1 (previously referee 3) does not need to be addressed, given that you have used more mice for the new experiments that support and strengthen the study. The second concern of referee 1 does also not need to be addressed experimentally, but the issue of which cell types mediate the effect should certainly be discussed.

Regarding referee 2's (previously referee 1) concerns, we agree that these are relevant to strengthen the main hypotheses of your study and therefore should be addressed. Both concerns relate to the requested rescue experiments that we decided are one of the 2 crucial issues that needed to be addressed during revision. While you show that the 2 identified miR-9-5p targets at least partially mediate the anti-fibrotic effect of miR-9-5p, their effect on TGFbeta signaling has not been investigated. I agree that fully addressing this point is probably a lot of work but referee 2 suggests one straight-forward experiment that we think should be performed (or an equivalent of it).

I also would like to mention that we usually do not allow 2 rounds of revision, but in this case I think that it was not very clear from the beginning what the revisions should entail, and I therefore think that you should be given a chance to address these 2 outstanding concerns. Please let me know if you have any questions.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

Fierro-Fernandez and colleagues report the anti-fibrogenic actions of miR-9-5p. The authors have included new experiments that have added to the rigor of the study and have increased my belief in the biology of these results as well as in the reasonable novelty of the findings. However, a couple of concerns are still present, and are listed below.

Major Comments:

1. I appreciate the authors' willingness to increase the N per group in the new in vivo studies. However, the experiments that have N=4-5 mice/group still are not enough to allay the concerns of biologic variability, especially considering these experiments utilize pharmacologic dosing (and not genetic KO's) which increase the interindividual variability.

2. Oligonucleotide miR and miR inhibitor delivery in vivo: Although the authors have included proper controls to show appropriate lentiviral delivery, oligonucleotide delivery also has to be confirmed by an independent measure of oligonucleotide presence (not just experimental target gene modulation). Labeled oligos or antibodies that can track the specific chemistry of the oligos in situ have been used successfully in the past. Alternatively, flow cytometric isolation of fibroblasts to show specific delivery in this way would be even better, as it would confirm at least one pertinent cell type is involved in this biology in vivo.

Referee #2:

In the present study, the authors found that miR-9-5p down-regulates TβRII and NOX4 to suppress fibrogenic signaling. They further demonstrated a therapeutic effect of miR-9-5b using a bleomycin induced lung fibrosis model. Paradoxically, miR-9-5p is induced under situations where fibrosis is enhanced. Thus, the authors discuss that miR-9-5p can be a negative feedback regulator of fibrosis induced by TGF-β. This hypothesis was confirmed by experiments in which miRNA inhibitor-9-5b was used. Experimental data mostly support the authors' conclusion. However, I have two comments.

1) To demonstrate that miR-9-5b inhibits fibrogenic signaling through down-regulating TβRII and NOX4, the authors performed rescue experiments (Figure 3F and G). I am afraid that the effects were only partial.

When they exogenously expressed TβRII, Nox4, an important target gene of TGF-β in fibrogenesis, was still repressed by miR-9-5b. Thus there is no surprise that the rescue was partial. Similarly, when they exogenously expressed Nox4, TβRII was repressed. If TGF-b signaling independent of Nox4 is involved in induction of α-SMA, FN, and Col1α, rescue by Nox4 expression could be partial. Thus I recommend the authors to exogenously express both TβRII and Nox4 in rescue experiments.

2) Because there are many miRNAs that target components of the TGF-β signaling pathway. I feel it important to identify target gene(s) of miR-9-5b in suppression of TGF-β signaling, to attract readers of this journal. At least, the effect of exogenous TβRII on TGF-β signaling in the presence of miR-9-5b should be examined in Figure 4C and D.

1st Revision - authors' response 06 July 2015

Referee #1

Fierro-Fernandez and colleagues report the anti-fibrogenic actions of miR-9-5p. The authors have included new experiments that have added to the rigor of the study and have increased my belief in the biology of these results as well as in the reasonable novelty of the findings. However, a couple of concerns are still present, and are listed below.

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the concerns of biologic variability, especially considering these experiments utilize pharmacologic dosing (and not genetic KO's) which increase the interindividual variability.

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We thank the reviewer for his/her comments. Although we agree with the conceptual importance of each of them we have focused on the required experiments stated in the first revision, in agreement with the editor´s recommendations.

Referee #2

In the present study, the authors found that miR-9-5p down-regulates TβRII and NOX4 to suppress fibrogenic signaling. They further demonstrated a therapeutic effect of miR-9-5b using a bleomycin induced lung fibrosis model. Paradoxically, miR-9-5p is induced under situations where fibrosis is enhanced. Thus, the authors discuss that miR-9-5p can be a negative feedback regulator of fibrosis induced by TGF-β. This hypothesis was confirmed by experiments in which miRNA inhibitor-9-5b was used. Experimental data mostly support the authors' conclusion. However, I have two comments.

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As suggested by the reviewer we have now performed experiments over-expressing both TGFBR2 and NOX4 at the same time. To make sure our previous results showing effects of each individual protein were consistent and reproducible, we have also included data of new individual transfections in every experiment of this new set of data. Transfection with plasmids containing cDNAs for these proteins were done by using 3 µg of each one. We have reduced the quantity of both plasmids compared to the previous “rescue” experiments, from 5 to 3 µg, as we observed that this results in significantly less toxicity and cell death. As shown in Fig 3F, over-expression of TGFBR2 or NOX4 resulted in a reduction of the miRNA inhibitory effect. When both proteins were over-expressed, the % of inhibition related to miR-9-5p tended to be lower compared to over-expression of each protein separately but did not reach statistical significance. Please keep in mind that only additive effects and not synergic should be expected when different pathways are interfered. The fact that the “rescue” effect observed after over-expression of TGFBR2 and NOX4 was partial may be due to several reasons including suboptimal levels of transfection efficiency (~ 20-30%) and a certain degree of toxicity, observed especially in the transfection experiments with the three components (miR-9-5p and the two plasmids). Moreover we would like to call the reviewer´s attention to the fact that other reasons of different biological nature may contribute to explain this behaviour. Among them are the fact that TGF-β may signal through non-canonical pathways or the possibility that the response in the presence of endogenous levels of proteins was close to maximal, thus preventing to observe effects of important magnitude when over-expressed.

2) Because there are many miRNAs that target components of the TGF-β signaling pathway. I feel it important to identify target gene(s) of miR-9-5p in suppression of TGF-β signaling, to attract readers of this journal. At least, the effect of exogenous TβRII on TGF-β signaling in the presence of miR-9-5p should be examined in Figure 4C and D.

As suggested by the reviewer, to demonstrate that miR-9-5p interferes with the TGF-β signaling pathway "de facto", we explored the archetypal effector of TGF-β, Smad2 as it was previously shown in Fig. 4D. As shown in the new Fig. 4F, exogenous expression of TGFBR2 induced a significant decrease in the miR-9-5p inhibitory effect on Smad2 phosphorylation, suggesting that, at least in part, this effect is mediated by TGFBR2.

2nd Editorial Decision 13 July 2015

Thank you for the submission of your revised manuscript to our journal. We have received the comments from referee 2, who supports publication of your study now, and we can therefore in principle accept it.

There are only a few modifications required, as explained below.

I noticed that the manuscript does not use our numbered reference style. Can you please change it? You can find the exact style in our author guidelines online. I also could not find the definition of the scale bars for figures 6B and 8B, can you please add it? The legend for E1 does not specify "n" , and for E8 it is not clear whether all images are shown at equal magnifications. Please add one sentence to explain.

EMBO press integrates supplementary figures in the main text now. They expand inline when clicked. However, we are currently in a transition phase and can only do this for 5 of the supplementary figures. Can you therefore please chose the 5 most important supplementary figures and rename them expanded view figures EV1, 2, etc? The legends for these need to stay in the main manuscript file. The remaining supplementary figures and tables will go into the Appendix file, together with their legends. Please label them figure S1, 2, etc. and table S1, 2, etc. Please also double check that they are mentioned in the main text with the correct name.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #2:

The authors have addressed my concerns. I have no additional comments.

2nd Revision - authors' response 17 July 2015

Thank you very much for notifying us of the pre-acceptance of manuscript EMBOR-2015-40750. We have now attended the recommendations of your mail (July 13) as follows:

1. We have performed all the modifications that you kindly suggested:

- We have adjusted the reference style to EMBO reports.

- The scale bars of figures 6B and 8B are described in the respective figure legends.

- The “n” in the figure legend of E1, now EV1, is shown.

- All images in E8, now EV4, are shown at equal magnifications and this is indicated in the figure legend.

2. Also, five figures have been selected to be converted to Expanded View Figures and another five are part of the Appendix file. All of them are correctly labeled.

3rd Editorial Decision 20 July 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

