EMBO reports

EphrinB2/EphB4 signaling regulates non-sprouting angiogenesis by VEGF

Elena Groppa, Sime Brkic, Andrea Uccelli, Galina Wirth, Petra Korpisalo-Pirinen, Maria Filippova, Boris Dasen, Veronica Sacchi, Manuele Giuseppe Muraro, Marianna Trani, Silvia Reginato, Roberto Gianni-Barrera, Seppo Ylä-Herttuala, Andrea Banfi

Review timeline:

Submission date:	23 August 2017
Editorial Decision:	25 September 2017
Revision received:	2 February 2018
Editorial Decision:	19 February 2018
Revision received:	3 March 2018
Accepted:	8 March 2018

Editor: Martina Rembold

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 25 September 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings and consider the data in large parts also solid. Reading through the reports it appears however that there are two major concerns. 1) The molecular link between ephrinB2/EphB4 and VEGF/ERK remains unclear. 2) The clinical relevance of the findings is limited and the data for this aspect are not fully convincing (Figure 8).

I think the first concern can be addressed to some extent by the experiments suggested by referee 1. Evaluating the effect of an ERK inhibitor and a second assay on VEGFR2 internalization can strengthen the data. Also the measurement of vessel diameter and the CD31 stainings should be improved as outlined by referee 1 and 2.
Apart from these experiments it will be crucial to strengthen the clinical relevance of the findings before the study becomes acceptable for EMBO reports. Apart from the analysis of a larger sample size, as outlined by referee 2, further data should be provided to significantly strengthen this aspect, as also emphasized and outlined by referee 3.

Given the overall constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Please note that all materials and methods should be included in the main manuscript file and may not be part of the Supplementary material.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors face the enigmatic problem of vascular intussusception showing that this mechanism is sustained by VEGF when delivered to ischemic muscle and regulated by ephrinB2/EphB4 axis. They show that ephrinB2/EphB4 does not interfere in VEGFR2 activation but modulates ERK. Then the authors exploit these results in term of therapeutic approach showing that pharmacological trigger of EphB4 normalize the abnormal vascular proliferation induced by VEGF gene delivery. In my opinion this a solid paper in particular for the in vivo data, but the molecular link between the role of ephrinB2/EphB4 and VEGF-stimulated ERK is not clear and not properly discussed.

Criticisms.
The in vivo data to illustrate the intussusception effect of VEGF are based on measurement of vessel diameters. However it important to measure the number of splitting cells along the different

treatment.

Because the authors demonstrate that ephrinB2/EphB4 regulates ERK phosphorylation it is important to evaluate the effect of an ERK inhibitor. Does it counteract the effect of sEphB4? Does an Erk activating mutant mimic the effect of EphrinB2-Fc?

I suggest to add a second assay to analyse VEGFR2 internalization using, for instance, the membrane biotinylation of VEGFR2. This assay implemented by specific inhibitors can give more information on dynamics receptor trafficking (endocytosis and recycling to plasmamembrane), that could be affected by Ephrin system as reported in the two papers quoted in the MS (Nature 465: 483-486, 2010; Nature 465: 487-491, 2010).

The in vitro data in Fig 6 show that ephrinB2/EphB4 controls EC proliferation induced by FGF2. Since this molecule can be involved in intussusception (e.g.Microscop Res Tech, 66:275, 2005) , it is important to discriminate this point by using at least one of the invivo models used to analyse the effect of low VEGF.

Referee #2:

This is a very interesting paper that invests deeply into dissecting the in vivo molecular mechanisms of VEGF-driven vessel formation. They show that EphB4 modulates Erk signaling and fine tunes the degree of EC proliferation induced by physiological VEGF levels in vivo. Although the clinical relevance of the work is not entirely robust it does give a flavor of future experiments that may test the utility of this approach.

Figure 1

The staining done here is very comprehensive and I applaud the authors of the 4 color approach. However, and this may be because I am not totally familiar with blood vessels in muscle, but it is hard to appreciate that the CD31 staining really defines actual blood vessels and not just 'strings' of cells. Perhaps inclusion of lower magnification images would help. Perhaps also DAB-stained Cd31 with a haematoxylin counter stain would be good so that we could at least see red blood cells in vessels. This becomes rather more important especially when we see the very aberrant vessels in v-low 3b image. Is there any way of quantitation these changes in morphology that could be included?

Figure 2
Please can we have some quantitation of the aberrant organization of the NG2, laminin and aSMA that the authors elude to in Fig 2 c.

Figure 3
Figure 3A-B and D-E again here. Although these images really do look beautiful it is hard to see exactly what is a vessel rather than a clump of CD31-positive cells, and especially how the authors determined where the lumen are in order to measure vessel diameter.

Figure 5
The quality of the vessel images here are convincing and clear.

Figure 8 C and E
Although statistically significant difference are reported do the authors really thunk that such small changes could have a biological consequence. I would say that especially in Fig 8E the statistical difference does not really seem a robust interpretation of a biological difference and perhaps should not be claimed as such.

Figure 8H
The ultrasound data here seem to be extremely variable. I know that these data are not easy to report but do the authors think that repeating this with increased n-numbers would provide a more robust interpretation of the data.

Referee #3:

This manuscript presents a significant step-forward in our understanding of the mechanisms controlling the growth of blood vessels, in particular the interplay of the VEGF and ephrin/EphB4 signalling pathways in controlling this biological process. The manuscript is well written, the data are generally compelling and the findings are novel. The weakness of the manuscript relates to the translational potential of the findings. Issues that need to be addressed are listed below.

Major:
1. The Authors claim that the data in Figure 8 demonstrate the therapeutic potential of EphB4 simulation, by systemic ephrinB2-Fc treatment, in controlling undesired vascular responses of VEGF gene delivery and improving its efficacy. I agree these data show that ephrinB2-Fc can modify the effects of VEGF, however, the data do not convince me that the combination of VEGF and ephrinB2-Fc is beneficial. It is not clear how the results with this combination are better than the Ad-LacZ + Fc control, particularly with regard to Figures 8 F, G and H. In fact, the Authors do not provide any comparisons of Ad-hVEGF + ephrinB2-Fc versus Ad-LacZ + Fc in the text of Results dealing with these data; the only comparisons specified relate to Ad-hVEGF + Fc versus Ad-hVEGF + ephrinB2-Fc. The Authors need to carefully address these issues as they are central to the clinical relevance of the study that is emphasized in the last part of the abstract.

Minor:
2. Figures 2B and 3C require specific information about whether or not differences are statistically significant.

3. It is not clear how the two panels (i.e. left and right panels) of each pair differ in Figure 3 A to E and Figure 7E and G.

1st Revision - authors' response 2 February 2018

Referee #1:

The authors face the enigmatic problem of vascular intussusception showing that this mechanism is sustained by VEGF when delivered to ischemic muscle and regulated by ephrinB2/EphB4 axis. They show that ephrinB2/EphB4 does not interfere in VEGFR2 activation but modulates ERK. Then the authors exploit these results in term of therapeutic approach showing that pharmacological trigger of EphB4 normalize the abnormal vascular proliferation induced by VEGF gene delivery. In my opinion this is a solid paper in particular for the in vivo data, but the molecular link between the role of ephrinB2/EphB4 and VEGF-stimulated ERK is not clear and not properly discussed.
We would like to thank the Reviewer for his/her appreciation and we have addressed the raised criticisms below.

Criticisms.
The in vivo data to illustrate the intussusception effect of VEGF are based on measurement of vessel diameters. However it important to measure the number of splitting cells along the different treatment.
We agree with the Reviewer that evidence of splitting events is required to demonstrate intussusception. However, we have previously addressed this point in detail in published work (Gianni-Barrera et al. Angiogenesis 2013), where the gold-standard technique of vascular corrosion casting with scanning electron microscopy was used to visualize intussusceptive events by the formation of hallmark intraluminal pillars. There we could ascertain that both normal and aberrant angiogenesis induced by increasing doses of VEGF was initiated by an initial stage of circumferential enlargement followed by formation of intraluminal pillars with similar frequency. However, these failed to split the significantly larger vessels induced by high VEGF, allowing their continued growth into angioma-like structures. Here we report that EphB4 signaling modulates precisely the degree of initial vascular enlargement achieved, which regulates the switch between normal and aberrant angiogenesis without affecting subsequent pillar formation itself, but rather their chance of successfully completing vessel splitting. Therefore, in view of these considerations, as well as of the very time- and effort-intensive nature of corrosion cast analysis, we hope to have

convinced the Reviewer that it is preferable not to repeat these experiments. We have now better clarified this point in the Discussion (page 19, second paragraph).

Because the authors demonstrate that ephrinB2/EphB4 regulates ERK phosphorylation it is important to evaluate the effect of an ERK inhibitor. Does it counteract the effect of sEphB4? Does an Erk activating mutant mimic the effect of EphrinB2-Fc?
In order to address this important point we have performed new *in vivo* experiments with the selective and potent ERK1/2 inhibitor SCH772984. The results show that the switch from normal to aberrant angiogenesis caused by EphB4 inhibition requires pERK1/2 activity and can be completely prevented by its inhibition, strengthening the conclusions of the manuscript (new Figure 7, Results pages 15-16 and Discussion page 21, last paragraph).

I suggest to add a second assay to analyse VEGFR2 internalization using, for instance, the membrane biotinylation of VEGFR2. This assay implemented by specific inhibitors can give more information on dynamics receptor trafficking (endocytosis and recycling to plasmamembrane), that could be affected by Ephrin system as reported in the two papers quoted in the MS (Nature 465: 483-486, 2010; Nature 465: 487-491, 2010).
We have followed the Reviewer's advice and investigated VEGF-R2 internalization by a proteolytic cleavage protection assay with Western blot analysis. The results confirmed that ephrinB2-Fc treatment does not impair VEGF-R2 internalization upon VEGF stimulation (Appendix Figure S4 and page 14, first paragraph).

The in vitro data in Fig 6 show that ephrinB2/EphB4 controls EC proliferation induced by FGF2. Since this molecule can be involved in intussusception (e.g. Microscop Res Tech, 66:275, 2005), it is important to discriminate this point by using at least one of the in vivo models used to analyse the effect of low VEGF.
We would like to thank the Reviewer for this suggestion. We have analyzed expression levels of *Fgf2* and *Vegfa* 3 days after implantation of V-low cells alone or with EphB4 blockade and we found that *Fgf2* expression did not change in the transition from normal to aberrant angiogenesis by EphB4 inhibition, while its level of upregulation during VEGF-induced angiogenesis was 3-/6-fold lower than the increase in *Vegfa* (Figure EV5 and page 13, second paragraph).

Referee #2:

This is a very interesting paper that invests deeply into dissecting the in vivo molecular mechanisms of VEGF-driven vessel formation. They show that EphB4 modulates Erk signaling and fine tunes the degree of EC proliferation induced by physiological VEGF levels in vivo. Although the clinical relevance of the work is not entirely robust it does give a flavor of future experiments that may test the utility of this approach.
We would like to thank the Reviewer for his/her appreciation.

Figure 1
The staining done here is very comprehensive and I applaud the authors of the 4 color approach. However, and this may be because I am not totally familiar with blood vessels in muscle, but it is hard to appreciate that the CD31 staining really defines actual blood vessels and not just 'strings' of cells. Perhaps inclusion of lower magnification images would help. Perhaps also DAB-stained Cd31 with a haematoxylin counter stain would be good so that we could at least see red blood cells in vessels. This becomes rather more important especially when we see the very aberrant vessels in v-low 3b image. Is there any way of quantitation these changes in morphology that could be included?
Figure 3
Figure 3A-B and D-E again here. Although these images really do look beautiful it is hard to see exactly what is a vessel rather than a clump of CD31-positive cells, and especially how the authors determined where the lumen are in order to measure vessel diameter.
These points are related, so we addressed them together. First we should highlight that unfortunately red blood cells cannot be visualized because they have been flushed out of the circulation, as tissues have been perfused in order to fix vessels in the open state to prevent collapse and quantify diameters accurately. Indeed, diameter quantification is the most accurate objective parameter of the morphological changes described, especially when presented as the distribution of several hundred

individual diameters in 1-µm incremental classes (e.g. in Figures 1 and 2), which allows the identification of specific sub-populations of aberrantly enlarged angioma-like structures.
On the other hand, in order to clarify the issue raised by the Reviewer and to further validate the nature of the observed vascular structures, we have performed immunostainings to detect functional polarization of endothelium. Expression of the lumen-specific marker podocalyxin, enveloped into the basal-specific marker laminin confirmed the correct apico-basal polarization of vascular endothelium (Appendix Figure S1, page 7, second paragraph and page 8, first paragraph). To this end, it is useful to notice that positive staining by intravascular lectin perfusion also confirms that observed endothelial structures indeed represent functionally perfused vessels, connected to the systemic circulation, rather than mere cellular aggregates (Figure EV2 and page 7, second paragraph).
Regarding the technical aspects of identifying lumens for diameter measurement, images used for quantification were kept with more background fluorescence, which clearly showed the muscle fibers and tissue around vessels, as opposed to the completely empty lumens.

Figure 2
Please can we have some quantitation of the aberrant organization of the NG2, laminin and αSMA that the authors elude to in Fig 2 c.
We have now quantified vascular coverage with NG2+ and SMA+ mural cells (new Figure 1F and page 7, first paragraph), confirming that the treatment with sEphB4 caused a switch from αSMA-negative pericytes to αSMA-positive smooth muscle cells.

Figure 5
The quality of the vessel images here are convincing and clear.
We would like to thank the Reviewer for his/her appreciation.

Figure 8 C and E
Although statistically significant difference are reported do the authors really think that such small changes could have a biological consequence. I would say that especially in Fig 8E the statistical difference does not really seem a robust interpretation of a biological difference and perhaps should not be claimed as such.
We would like to thank the Reviewer for pointing out this important aspect. In fact, upon careful reconsideration of all images and corresponding quantifications, we realized that in ischemic tissues vessel diameters provided an unreliable measure of the size differences clearly observable on histological sections. In fact, contrary to normal tissues, ischemic muscles are perfused erratically by fixative during sacrifice by perfuse-fixation and this leads to variable degrees of collapse of the vascular structures, whereas in normal tissues essentially all vessels are fixed in their physiologically open size. Since diameters are always measured as the shortest segment, vessel collapse severely affects quantification of this parameter, as well as of vessel areas. On the other hand, vessel perimeters are much less affected by vascular collapse. Therefore, we quantified both old and new images from ischemic tissues by measuring vessel perimeters and the new data show robust differences between groups, which more accurately reflect the observable morphologies (new Figure 8E and page 17, last paragraph/page 18, first paragraph).

Figure 8H
The ultrasound data here seem to be extremely variable. I know that these data are not easy to report but do the authors think that repeating this with increased n-numbers would provide a more robust interpretation of the data.
We have repeated an ischemia experiment and increased the group size (now n is 7-10 mice/group at day 11). However, variability remained high and differences between treatment groups were not statistically significant. Therefore, we agree with the Reviewer that implications about the therapeutic potential of this approach are premature. Accordingly, we now conclude that EphB4 stimulation is effective in ensuring normal angiogenesis despite uncontrolled VEGF gene delivery by a clinically relevant adenoviral vector, both in normal tissue and in ischemic muscle, in conditions where a variety of endogenous angiogenic pathways are up-regulated and tissue repair is occurring. However, its therapeutic potential remains to be established and will require further investigation in optimized experiments (Abstract, Results pages 17, last paragraph, and 18 and Discussion page 23, last paragraph).

Referee #3:

This manuscript presents a significant step-forward in our understanding of the mechanisms controlling the growth of blood vessels, in particular the interplay of the VEGF and ephrin/EphB4 signalling pathways in controlling this biological process. The manuscript is well written, the data are generally compelling and the findings are novel.
We would like to thank the Reviewer for his/her appreciation.

The weakness of the manuscript relates to the translational potential of the findings. Issues that need to be addressed are listed below.

Major:
1. The Authors claim that the data in Figure 8 demonstrate the therapeutic potential of EphB4 simulation, by systemic ephrinB2-Fc treatment, in controlling undesired vascular responses of VEGF gene delivery and improving its efficacy. I agree these data show that ephrinB2-Fc can modify the effects of VEGF, however, the data do not convince me that the combination of VEGF and ephrinB2-Fc is beneficial. It is not clear how the results with this combination are better than the Ad-LacZ + Fc control, particularly with regard to Figures 8 F, G and H. In fact, the Authors do not provide any comparisons of Ad-hVEGF + ephrinB2-Fc versus Ad-LacZ + Fc in the text of Results dealing with these data; the only comparisons specified relate to Ad-hVEGF + Fc versus Ad-hVEGF + ephrinB2-Fc. The Authors need to carefully address these issues as they are central to the clinical relevance of the study that is emphasized in the last part of the abstract.
We agree with the Reviewer's comment. We have performed new experiments to increase the group sizes and have now included comparisons with Ad-LacZ treatments in Figure 8E and in the text. We have realized that in ischemic tissues vessel diameters provided an unreliable measure of the size differences clearly observable on histological sections, due to erratic perfusion of fixative and variable degrees of vessel collapse (see also response to Reviewer 2, Figures 8C and E). Therefore, we quantified both old and new images from ischemic tissues by measuring vessel perimeters and the new data show robust differences between groups (including Ad-LacZ), which more accurately reflect the observable morphologies (new Figure 8E and page 17, last paragraph/page 18, first paragraph).
Regarding functional data (perfusion and tissue necrosis/regeneration), variability remained high and observed trends towards improvement were not statistically significant. In particular, it should be noticed that the Ad-LacZ control group already shows limited necrosis (10%) and robust regeneration (55%) at day 11, although the use of aged hyperlipidemic mice significantly dampens spontaneous regeneration compared to wild-type strains. This highlights the need to carefully evaluate the therapeutic potential of such approach in carefully designed ad hoc studies, possibly in more pre-clinically relevant large animal models. Therefore, we have removed all claims about a potential therapeutic benefit. We now conclude that EphB4 stimulation is effective in ensuring normal angiogenesis despite uncontrolled VEGF gene delivery by a clinically relevant adenoviral vector, both in normal tissue and in ischemic muscle, in conditions where a variety of endogenous angiogenic pathways are up-regulated and tissue repair is occurring. However, its therapeutic potential remains to be established and will require further investigation in optimized experiments. We have therefore modified the Abstract, Results (pages 17, last paragraph, and 18) and Discussion (page 23, last paragraph) accordingly.

Minor:
2. Figures 2B and 3C require specific information about whether or not differences are statistically significant.
Quantifications of mean diameter sizes and corresponding statistical analyses have now been included in new panels in Figures 1D (page 6, last paragraph), 2C and 2F (page 8, first paragraph and page 9, first paragraph).

3. It is not clear how the two panels (i.e. left and right panels) of each pair differ in Figure 3 A to E and Figure 7E and G.
We have now provided color-coded labels under each image to clarify which staining is depicted in each case (new Figures 2A, 2D, 6E and 6G).

2nd Editorial Decision 19 February 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are positive about the study and support publication in EMBO reports without further revision.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the acceptance of your study.

- Please update the references to match the numbered style of EMBO reports. The respective EndNote file is available for download from our Guide to Authors (https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view).

- On page 10 you mention Figure 4E. I think that this paragraph should refer to Fig. 3E

- Please provide a scale bar for Fig. 7E.

- Please define the size of the scale bars in the legend of Figure S1.

- Appendix Figure S2: Please add a definition of the asterisk in the right image to the figure legend.

- Please complete the section on author and manuscript information in the upper left corner of the Author Checklist.

- Our data editors from Wiley have already inspected the figure legends for completeness. Please find their suggested changes in the attached Word file.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors did a good revision of the MS to properly reply to my comments

Referee #2:

The authors have satisfied my concerns in their revised MS

Referee #3:

The Authors have satisfactorily and thoughtfully addressed my concerns about the manuscript.

2nd Revision - authors' response 3 March 2018

In the new submitted files we have made the corrections you had identified, as detailed in the point-by-point response below.

Please update the references to match the numbered style of EMBO reports. The respective EndNote file is available for download from our Guide to Authors
The references have been updated according to the correct style.

On page 10 you mention Figure 4E. I think that this paragraph should refer to Fig. 3E
The mistake figure reference has been corrected.

Please provide a scale bar for Fig. 7E.
A new Fig. 7 file has been uploaded with the missing scale bar and its description has been added to the corresponding legend.

Please define the size of the scale bars in the legend of Figure S1.
The scale bar definition has been added.

Appendix Figure S2: Please add a definition of the asterisk in the right image to the figure legend.
The missing information has been added to the figure legend, as well as to those for Figures 1, 6, 7 and S1, where asterisks were also shown on IF panels, but were not described in the text.

Please complete the section on author and manuscript information in the upper left corner of the Author Checklist.
A completed Author Checklist file has been uploaded.

Our data editors from Wiley have already inspected the figure legends for completeness. Please find their suggested changes in the attached Word file.
We are fully in agreement with the suggested changes.

Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.
A Synopsis text (Summary + bullet points) has been uploaded together with a graphical abstract file

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andrea Banfi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45054V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen basing on common practice in the field and previous experience with similar analyses.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See the relevant section for each experiment in the Material and Methods .
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were randomly assigned to experimental groups with equal representation of both sexes (for details, see the relevant section for each experiment in the Material and Methods).
For animal studies, include a statement about randomization even if no randomization was used.	Statements have been included in the Materials and Methods (page 35, first paragraph; page 27, third paragraph).
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Group allocation was randomized in each experiment (page 35, first paragraph; page 27, third paragraph). All histological analyses were performed by blinded investigators (page 31, second paragraph; page 36, second paragraph).
4.b. For animal studies, include a statement about blinding even if no blinding was done	Statements have been included in the Materials and Methods (page 31, second paragraph; page 36, second paragraph)
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Normal distribution was tested with Shapiro-Wilk normality test. If distribution was normal, parametric test was applied; if not, non-parametric equivalents were employed (see "Statistics" section in the Materials and Methods page 36 and figure legends).
Is there an estimate of variation within each group of data?	Yes: all data are presented as mean +/- SEM. For n<5, individual data points are shown in the graphs.
Is the variance similar between the groups that are being statistically compared?	We did not assume equal variance during statistical testing.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody details and providers are provided in the Material and Methods (page 30, page 32, page 33, page 34 and page 36).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Details are provided in the Material and Methods in the relevant chapter for each experiment (page 27, page 28 and page 34).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	See "Study approval" section in Materials and Methods (page 37)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance with the ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA