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Rap1 promotes endothelial mechanosensing complex formation, NO release and normal endothelial function

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

Editor: Nonia Pariente

1st Editorial Decision 19 December 2014

Thank you for your submission to EMBO reports and please accept my apology for the time it has taken us to contact you with a decision on your study. It was sent to three referees and we have received the reports form two of them. I have decided to make a decision based on them to avoid any further loss of time. As you will see, although both referees find the topic of interest, they have concerns with the quality and conclusiveness of some of the data and importantly consider that additional insights into the role of Rap1 would be needed for publication in EMBO reports.

Given that both referees provide constructive suggestions on how to make the work more conclusive and provide some evidence for the role of Rap1, I would like to give you the opportunity to revise your manuscript. Please note that we do agree with referee 1 that finding a Rap GEF would be out of the scope, but assessing the role of Epac would be required. We would also require insight into Rap1 function in this context and not toning down of the message as referee 1 mentions.

If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #2:

In this paper, Lakshmikanthan et al present two data sets on the role of Rap1 in endothelial cells, i.e. a series of elegant in vivo experiments, complemented by in vitro studies.
Their concluding model proposes that Rap1 is activated downstream of PECAM1 in response to shear, signaling towards a VEcad- VEGFR complex which in turn activates PI3K, Akt and eNOS, leading to NO production and vascular stability.

The majority of the data rely on loss of Rap1 experiments, both in vivo as well as in vitro. Most of these data are strongly suggestive for a role for Rap1 in shear-induced NO production and regulation of hypertension. At the same time however, the evidence for an active role for Rap1 in this pathway is less well developed.

Specific comments.

The analysis of vascular endothelial integrity as shown in Fig 1C and E1A are not convincing. The imaging should be improved as junctional organization is now hard to discern. Also the permeability data (now data not shown) should be included.

The p-eNOS blot in 3C is , despite the quantification , not very convincing. This may be due to high basal levels, which makes one wonder about the extent of activation imposed upon eNOS by the Rap1 pathway. The analysis / exposure of the blots should be improved

The data in 4A suggest the constitutive association of VE-cadherin with PI3K and the shear induced recruitment of VEGFR2 in the complex - this interpretation is not obvious from the text. Authors should comment on this.
Also: the IgG band appears to run at ~ 100 kD, which is unusual for denaturing PAGE. Loading controls for VEGFR2 are lacking.

In 4B, total levels for VEGFR2 as well as for PECAM1 are lacking

Similarly, in 4C PECAM-1 levels in the VEcad si samples should be included. The presentation of the blots is confusing - separate PECAM from VE cad panels in the blots under the bar graph.

Authors state that shear quickly and transiently activates Rap1 - these kinetic data should be shown and quantified. This is particularly relevant as the phospho-data in 3, 4A, B require 5-15 min of shear to detect signaling events. Although I realize that some events will be temporally distinct, this is a relevant point if the authors claim that activation of Rap1 is required for activation of the pathway.

In conclusion, the data that show direct activation of Rap1 as being instrumental in this pathway are limited and should be improved/extended to support the model. Alternatively, Rap1 could be proposed to be a basal, required activity for shear responsiveness, which would be the conclusion from the loss of Rap1 experiments. Ideally, a rap1 GEF would be identified downstream of PECAM, but this will require a significant amount of work. Epac could be simply ruled out using 007, which is relevant as activated Epac is membrane-recruited and could well regulate such a junctional complex in a Rap1 dependent fashion.

Referee #3:

This manuscript by Lakshimikanthan et al investigated the role of Rap1 in endothelial cell function

using endothelial specific Rap1 KO mice. The authors found that deletion of Rap1 decreases NO production and lead to hypertension. In addition, the authors found the crucial role of Rap1 in regulating shear stress-induced VEGFR2-PI3-K-Akt-eNOS signaling. The data are potentially interesting, but the data are somewhat descriptive, and need more details to determine the exact mechanistic insight of Rap1 in regulating VE-Cadherin-VEGFR2 complex. In addition, the quality of several data is not acceptable.

Major
1. Since the Rap1 KO did not show any effect on permeability can be very novel and important for this study, please show the actual data of permeability, which was described in the text as "data not shown".
2. Several quantification data did not fit well with the "representative" blots. For example, in fig. 3C it is very difficult to see 2-fold increase of p-eNOS in CA-Rap1 overexpressed samples (2nd lane from left) compared to basal level.
3. In several figures the authors divided the blot in several pieces. Since it is crucial to compare the intensity of each band in same blot, this is not acceptable. Therefore, Fig.4A (IgG), Fig.4B, and Fig.4C need to be corrected.
4. The authors stated that "Rap1 promotes association of PECAM-1 with VEGFR2", but the contribution of VE-Cadherin in this context is unclear. The inhibitory effect of Rap1 deletion on sheer stress-induced VEGFR2 tyrosine phosphorylation is more obvious than those in p-eNOS and Akt (Fig.3B). Is there any difference in VEGFR2 expression in Rap1b KO cells?
5. The possible role of Rap1 in regulating Rac1 has been reported1. Since Rac1 is crucial for NADPH oxidase activation2, is it possible that the deletion of Rap1 may have some effect on ROS production? If so, is it possible that Rap1-Rac1 mediated ROS production can explain the contribution of Rap1 in regulating VEGFR2 tyrosine phosphorylation?

Minor
1. In Fig. 4C, 2nd blots from the top, it was not clearly described which blots were WB PECAM-1 and VE-Cadherin.
2. It was not clearly stated how the authors generated shear stress to the cells.
3. It has been reported that the coating of the dishes can significantly alter EC responses to shear stress. Therefore, it is necessary to clarify the coating of the dishes3.

References

1. Stefanini, L., et al. Rap1-Rac1 circuits potentiate platelet activation. Arterioscler Thromb Vasc Biol 32, 434-441 (2012).
2. Ming, W., Li, S., Billadeau, D.D., Quilliam, L.A. & Dinauer, M.C. The Rac effector p67phox regulates phagocyte NADPH oxidase by stimulating Vav1 guanine nucleotide exchange activity. Mol Cell Biol 27, 312-323 (2007).
3. Collins, C., et al. Haemodynamic and extracellular matrix cues regulate the mechanical phenotype and stiffness of aortic endothelial cells. Nature communications 5, 3984 (2014).

1st Revision - authors' response 11 February 2015

We thank the Reviewers and are grateful for the insightful comments and helpful suggestions. We have performed additional experiments, as suggested by the Reviewers, including the kinetics of Rap1 activation in response to shear and excluded the role of Epac with 007. We have also addressed technical shortcomings of our previous submission. The revisions support our previous conclusions.

We have addressed all other concerns to the best of our ability, as outlined below. Resulting text edits are marked as “track changes” in the Word document. We have also re-organized the figures to conform to the Journal’s requirements. We trust that the Reviewers will now find the revised MS appropriate for publication in EMBO Reports.

Response to Referee #2: We thank the Reviewer for finding our studies elegant.

Specific comments:

"The analysis of vascular endothelial integrity as shown in Fig 1C and E1A are not convincing. The imaging should be improved as junctional organization is now hard to discern.(…)"

Response: We have now obtained higher resolution images and replaced the original panels in Fig. 1C and E1A. These new data are consistent with our previous conclusion that PECAM-1 organization in Rap1-ECKO aortae is normal.

"(…)Also the permeability data (now data not shown) should be included."
Response: These data are now shown in Figure 1C.

"The p-eNOS blot in 3C is , despite the quantification , not very convincing. This may be due to high basal levels, which makes one wonder about the extent of activation imposed upon eNOS by the Rap1 pathway. The analysis / exposure of the blots should be improved"

Response: The anti-phospho-eNOS monoclonal antibody used for this blot stains two bands, the identity of which has been debated in the literature. Importantly, (Fleming et al., 2001) address that issue and demonstrate that the top band of the doublet detected by this antibody is non-specific (now indicated in revised fig. 3C). Therefore, we focused our analysis on the lower, eNOS/P-Ser1177-specific band. Low intensity of that band in untreated samples is consistent with low basal level of phospho-eNOS.

"The data in 4A suggest the constitutive association of VE-cadherin with PI3K and the shear induced recruitment of VEGFR2 in the complex - this interpretation is not obvious from the text. Authors should comment on this. (…)"

Response: We thank the Reviewer for pointing this out; we have now included this interpretation in the text on p. 5 of the revised MS.

"(…)Also: the IgG band appears to run at ~ 100 kD, which is unusual for denaturing PAGE. Loading controls for VEGFR2 are lacking."

Response: We apologize for incorrectly marking what is a non-specific band (now marked with an asterisk and described in the revised figure legend) as an IgG band. Loading controls for VEGFR2 are now included in the revised panel 4A.

"In 4B, total levels for VEGFR2 as well as for PECAM1 are lacking"
Response: The blots shown in panels 4A and 4B were obtained from the same experiment. Protein lysates obtained in this experiment were equally divided and processed; one half of the lysates were immunoprecipitated with p85 antibody (Figure 4A) and the other half – with PECAM antibody (Figure 4B). Total VEGFR2, PECAM1 and actin levels in protein lysates are now shown in Figure 4A as loading controls for panels, 4A and 4B. This information is now indicated in the revised figure legend.

"Similarly, in 4C PECAM-1 levels in the VEcad si samples should be included. The presentation of the blots is confusing - separate PECAM from VE cad panels in the blots under the bar graph."

Response: PECAM-1 levels in the VEcad si samples are now included in panel 4C. We apologize for the confusing formatting of the figure. We have now revised it for clarity.

"Authors state that shear quickly and transiently activates Rap1 - these kinetic data should be shown and quantified. This is particularly relevant as the phospho-data in 3, 4A, B require 5-15 min of shear to detect signaling events. Although I realize that some events will be temporally distinct, this is a relevant point if the authors claim that activation of Rap1 is required for activation of the pathway."

Response: We have performed such kinetic analysis of Rap1 activation, showing Rap1 activation at 1 min and maximum at 5 min, which precedes maximum pAkt1 and p-eNOS, occurring at 15 min. These data are now shown and quantified in a new Figure E3A.

"In conclusion, the data that show direct activation of Rap1 as being instrumental in this pathway are limited and should be improved/extended to support the model. (…)Epac could be simply ruled out using 007, which is relevant as activated Epac is membrane-recruited and could well regulate such a junctional complex in a Rap1 dependent fashion."

Response: the new kinetic data shown in new figure E3A, as discussed above, further support direct activation of Rap1 as instrumental in this pathway. As suggested by the Reviewer, to rule out Epac as the Rap1 GEF responsible for its activity promoting eNOS activation, we examined the effect of 007 on eNOS activation (eNOS/P-Ser1177 induction) in WT ECs. We found that while 007 treatment of ECs induced Rap1 activation, as expected, it did not lead to activation of eNOS. Therefore, Epac is not the GEF responsible for Rap1 activity promoting eNOS activation. We included these data in new Figure E3B.

Response to Referee #3: We thank the Reviewer for finding our data interesting.

Major

"1. Since the Rap1 KO did not show any effect on permeability can be very novel and important for this study, please show the actual data of permeability, which was described in the text as "data not shown"."

Response: These data are now shown in Figure 1C.

"2. Several quantification data did not fit well with the "representative" blots. For example, in fig. 3C it is very difficult to see 2-fold increase of p-eNOS in CA-Rap1 overexpressed samples (2nd lane from left) compared to basal level."

Response: The anti-phospho-eNOS monoclonal antibody used for this blot stains two bands, the identity of which has been debated in the literature. Importantly, (Fleming et al., 2001) address that issue and demonstrate that the top band of the doublet detected by this antibody is non-specific (now indicated in revised fig. 3C). Therefore, we focused our analysis on the lower, eNOS/P-Ser1177-specific band. Low intensity of that band in untreated samples is consistent with low basal level of phospho-eNOS.

"3. In several figures the authors divided the blot in several pieces. Since it is crucial to compare the intensity of each band in same blot, this is not acceptable. Therefore, Fig.4A (IgG), Fig.4B, and Fig.4C need to be corrected."

Response: We apologize for the confusing presentation of data in these panels; in the original submission we compared the intensity of each band in the same blot. We have now edited the figures to show that uncropped blots were used for panels 4A and 4B. Black lines in the blots indicate where the PVDF membranes were cut for blotting with the specific antibodies, as indicated. We also edited Fig. 4C to indicate that no cuts were made in the PECAM and VE-Cadherin blots (between control and experimental siRNA conditions).

"4. The authors stated that "Rap1 promotes association of PECAM-1 with VEGFR2", but the contribution of VE-Cadherin in this context is unclear. The inhibitory effect of Rap1 deletion on sheer stress-induced VEGFR2 tyrosine phosphorylation is more obvious than those in p-eNOS and Akt (Fig.3B). Is there any difference in VEGFR2 expression in Rap1b KO cells?"

Response: Our data in figure 4A suggest that VE-Cadherin forms a constitutive complex with p85, which is unchanged by shear or Rap1-deficiency and that shear stress induces VEGFR2 recruitment to this complex. We have now revised the text of the MS (on p.5) to reflect that conclusion. We find that while the differences in eNOS and Akt phosphorylation between WT and KO cells may be less pronounced than that of VEGFR2, they are consistently and significantly lower in KO cells. We examined expression of all components of the complex, including VEGFR2, and found it is unchanged in Rap1b KO cells, as now indicated in Fig. 3B.

"5. The possible role of Rap1 in regulating Rac1 has been reported1. Since Rac1 is crucial for NADPH oxidase activation2, is it possible that the deletion of Rap1 may have some effect on ROS production? If so, is it possible that Rap1-Rac1 mediated ROS production can explain the contribution of Rap1 in regulating VEGFR2 tyrosine phosphorylation?"

Response: We agree with the
Reviewer that Rap1 may affect ROS production and that Rac1 may be an intermediary of that interaction. Interestingly, we find that in Rap1b-KO ECs, basal level of Rac1 activity is lower than that in WT ECs (see Figure 1 for Reviewers).
(data not shown)
Such lower Rac1 activity would be expected to lead to lower NADPH oxidase activation and lower ROS generation. However, we also found, and reported last year (Wang et al., 2014) that inhibition of Rap1 leads to increased ROS formation, while, conversely, activation of Rap1 – decreases ROS. Therefore, the link between Rap1, Rac and NADPH oxidase(s) appears complex and requires a separate, systematic study.

Minor
"1. In Fig. 4C, 2nd blots from the top, it was not clearly described which blots were WB PECAM-1 and VE-Cadherin."

Response: We apologize for the lack of clarity. We have reorganized this panel and revised the legend of the blot in Fig. 4C accordingly.

"2. It was not clearly stated how the authors generated shear stress to the cells."

Response: Shear stress was generated using a cone viscometer. This information is now also included in the "Results and Discussion" section on p. 5.

"3. It has been reported that the coating of the dishes can significantly alter EC responses to shear stress. Therefore, it is necessary to clarify the coating of the dishes3."

Response: We agree with the Reviewer. For analysis of NO release, mouse ECs were initially plated on collagen and cultured for up to 48 hours to reach confluence. For analysis of mechanosignaling, human ECs were transfected with siRNA, and then also cultured for 48h to obtain confluency prior to induction of quiescence and shear stress. It is assumed that in both cases, during the time in culture, ECs produce their own ECM, of which fibronectin is the predominant component. To indicate the fact that not a specific ECM was used and that cells were cultured, we revised the text of Fig. 3 legend on p.10 accordingly.

REFERENCES

Fleming, I., B. Fisslthaler, S. Dimmeler, B.E. Kemp, and R. Busse. 2001. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circulation Research*. 88:E68-75.

Wang, H., Y. Jiang, D. Shi, L.A. Quilliam, M. Chrzanowska-Wodnicka, E.S. Wittchen, D.Y. Li, and M.E. Hartnett. 2014. Activation of Rap1 inhibits NADPH oxidase-dependent ROS generation in retinal pigment epithelium and reduces choroidal neovascularization. *The FASEB Journal*. 28:265-274.

2nd Editorial Decision 24 February 2015

Thank you for your patience while we have reviewed your revised manuscript. It was seen by referees 2 and 3, whom -as you will see from the reports below- are both positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Please address the issue with figure 1E mentioned by referee 2

- We now encourage the publication of original source data -particularly for electrophoretic gels and

blots, but also for graphs and microscopy images- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #2:

The authors have adequately addressed all my previous concerns. I have no additional comments except one minor issue:

in Fig 1E top left panel , the inset is not a zoom of the larger image.

I congratulate the authors with this interesting and elegant study.

Referee #3:

The paper is now acceptable.

2nd Revision - authors' response 28 February 2015

We would like to thank you and the Referees for the positive reception of our revised manuscript and finding it appropriate for publication in EMBO Reports. We have addressed the minor issues and made the requested corrections, as follows:

• We have corrected Figure 1E and legend;
• We are including source data for all our blots as PDF files that contain the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet with the data behind the graphs.

Again, thank you very much for your assistance in publishing our paper.

3rd Editorial Decision 02 March 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.

