EMBO reports

PAR-3 controls endothelial planar polarity and vascular inflammation under laminar flow

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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.)

1st Editorial Decision 30 October 2017

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

As you will see, while the referees agree that the study is potentially interesting, they also all indicate that it requires significant revision to substantiate the findings. Moreover, the referees point out that in many cases the description of the findings is either unclear or not in agreement with the data and in some places also confusing. Moreover, the referees point out missing control experiments.

From the referee comments it is clear that, as it stands, publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, and the constructive comments from the referees, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

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, please ensure to sepcify 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 each respective figure legend. 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.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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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 or if you require more time.

REFEREE REPORTS
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Referee #1:

This manuscript analyzes the role of Par3 in endothelial responses to flow. They report that deletion of Par3 reduces EC sensitivity to flow such that higher flow is required to induce golgi orientation. Deletion of Par3 also reduces GSK3b inhibitory phosphorylation on Ser9, thus GSK3b is more active in Par3-/- cells, which leads to increased NF-kB and atherosclerosis. These effects involve formation of 2 distinct complexes, a GSK3-aPKC complex that is induced by Rho/ROCK, and a Par3-aPKC complex that is inhibited by Rho/ROCK. The Par3-aPKC complex localizes to the cell front under flow, whereas the GSK3 complex localizes to the cell rear under flow, consistent with higher Rho activity in the rear. The overall idea then is that Par3 is involved in polarized inhibition of GSK3, which regulates both effects on golgi orientation and NF-kB/inflammation.

The manuscript uses sophisticated methods and examines arteries with great care, reporting a number of interesting results. The careful analysis of orientation in different parts of arteries and the dose-dependence of orientation are commendable. Unfortunately, the work is presented in such a confusing way that it is quite hard to follow. At various places in the paper they conclude that effects of Par3 on PCP mediate effects on inflammation, whereas in other places they say the opposite. The paper also contains numerous experimental or methodological flaws. In addition to addressing the technical issues, the authors must carefully think this through and present a well-organized, coherent story from the beginning.

Specific Criticisms:

1. Fig 1 does not convincingly show normal apical-basal polarity. 3D sections that actually reveal polarity are required to make this point.

2. Contrary to the text, fig 2b shows no failure to orient in flow in arteries or capillaries, and only a slight effect in veins. There is also slight effect at the sprouting front, despite the claim that it is unaffected. Fig 2D explains this better as a shift in sensitivity but the initial presentation of the data is problematic since the text does not match the data.

3. The increased NF-kB in the aorta from Par3-/- mice does not correlate with Golgi orientation. There is an increase even in the inner curvature region where orientation/alignment do not differ. So par3 effects on these variables appear to be independent. They eventually say something about this in the Discussion but the way the data are presented and analyzed leads one to believe that these 2 effects should be linked, which contributes to the confusion. For example, they conclude that "Thus, PAR-3 inhibits atherosclerosis onset by promoting endothelial PCP and blocking endothelial inflammation", which is not supported by the data.

4. Results in extended fig 3 need to be quantified. All of the Western blots throughout the paper need to be quantified from multiple experiments and statistics analyzed.

5. Plasma lipids need to be determined in WT and par3 iec apoE-/- mice on high fat diets.

6. The experiments with co-IP of GSK3b, Par3 and aPKC are the most mechanistic ones in the paper but effects of flow on these complexes was not analyzed. This is an important point that would link several of the observations in the study.

7. The NF-kB results in extended fig 5 are puzzling. They show that flow triggers a rapid decrease in nuclear p65 instead of an increase, as has been reported in many papers and also in Extended fig 8. The decrease is merely less after Par3 knockdown,

8. The experiments showing differential associations of Par3, aPKC and GSK3b in 293 cells needs

to be confirmed in endothelial cells. In this regard, it is puzzling that extended fig 6d,e analyzes a complex of GSK3 with VE-cadherin instead of with aPKC, which would correlate with Fig 4c.

9. Fig 5b. The quantification seems to show that in Par3KO cells, BIO increases golgi orientation in the marginal zone and sidewall. Is this effect significant?
Fig 5c is confusing. How can nuclear p65 be negative? Further, it shows nuclear p65 elevation in the sidewall instead of the inner curvature. Something must be mislabeled.
In any case, it would be helpful to indicate what is quantified on the Y axis in the figure.

10. The idea that loss of cell polarity in ECs leads to pathology is exactly opposite to the data showing that effects of Par3 and GSK3 on polarity and NF-kB are separable.

Minor Points

Planar cell polarity generally refers to non-canonical Wnt signaling, which has not been shown to mediate endothelial cell alignment or orientation in flow. Further, while alignment has been linked to inflammation (ref 40), golgi orientation (which is closer to traditional PCP) has not. Indeed, the cited references in the Introduction (5 and 6) never use the term PCP in discussing effects of flow on endothelial cells. The authors need to clarify these points and to be more careful about using these terms correctly.

It is not correct that "the role of PAR-3 in endothelial polarization in vivo has not been shown". See Liu et al J Cell Biol. 2013 Jun 10;201(6):863-73. Polarized formation of a number of protein complexes requires Par3.

p. 5. VE-cadherin forms a homophilic complex, not a hemophilic complex.

Fig 4a. previous work has reported flow induces phosphorylation of GSK3b on Ser9, which was linked to tubulin stability. See McCue Circ Res. 2006 Apr 14;98(7):939-46. And Biswas Am J Pathol. 2006 Jul;169(1):314-24..

It would be helpful to put extended fig 6b and fig 4c next to each other since they are complementary. Similarly, Fig 4f,g and extended 6d,e should be shown together.

The authors make the point in ther Discussion that "Unlike in epithelial cells, endothelial PAR-3 did not seem to be critical for the establishment of apical basal polarity and cell-cell junction formation". Actually, inhibition of par3 in epithelial cells generally gives rise to a partial defect in apical-basal polarity, slowing cell polarization but not necessarily blocking at later times. This may not be so different from the shift in sensitivity to flow magnitude reported here.

Referee #2:

This manuscript describes a series of observations with endothelial cells in vivo and in vitro. The observations are linked to each other via PAR3, which can associate with PAR6/aPKC to form a 'polarity complex'. The PAR complex in turn is well known to interact with GSK3β. The manuscript starts with a description of various phenotypes in endothelial cells in vivo that have been inducibly depleted of PAR3. It then goes on to study the effects of PAR3 depletion in cultured human endothelial cells, which includes a wide range of different results relating to a variety of other proteins. The main focus in the abstract is on GSK3β interaction with the PAR complex, but a number of other rather preliminary results on ROCK and a RhoA biosensor are included, as well as on NF-kB and microtubules. These data makes the manuscript rather unfocused, and it reads like a combination of several different projects. It would strongly benefit from focusing on a core message relating to the function of the PAR complex in endothelial cells.
The lack of page numbers means that I cannot refer to specific pages in the text in my comments below. In addition, a considerable amount of information is missing or does not tally between legends and text/methods.

Other major points:
1. How efficient is PAR3 depletion in mouse endothelial cells following tamoxifen administration?

It is essential to quantify this because inducible depletion can be very variable between models.
2. Figure 1: The sprouting defect of PAR3-depleted retina is not so obvious from the images and needs quantifying to know if it is similar to previous analysis. What is isolectin-B4 and what does it stain for? It is not mentioned in the text.
3. Figure 2: The full methodology for the quantification of retinal polarity and for estimating flow rates and flow direction needs to be provided, otherwise it is not possible to understand how the results were generated, or justify the conclusion that Par3 is only required at low to medium shear stress in vivo. The work-flow for the quantification should be shown in a supplementary figure, because it is very difficult to follow the method. Enlarged regions of the vessels should be shown in single colors so that the Golgi localization and how it is scored can be clearly understood. Are the vessels shown capillaries? Images should be expanded to show some of each vessel type and label each vessel type on the images. The effects on polarity of the Golgi appear fairly minor.
4. Extended Fig. 1b: the Erg staining does not appear restricted to the nucleus here, so it is difficult to see how the orientation of the Golgi with respect to the nucleus was determined.
5. Figure 3d/e: quantification of the Oil red area should be done in μm2 rather than pixels, to give the absolute area, and should also take into consideration the total area measured, because the aortas from PAR3-depleted mice could have a different size/area than control mice.
6. Extended Fig. 5: How many separate experiments were analysed for NF-kB nuclear localization? The authors state more than 100 cells were analysed, but in how many independent experiments? What happens at 24 h after flow initiation? Why is the NF-kB nuclear localization high in control cells before the onset of flow? It should be very low, since it is known that flow transiently increases NF-kB nuclear localization from published studies. Are the cells under pro-inflammatory conditions, which would induce its nuclear localization? The text referring to this figure is inaccurate, because NF-KB is in the nucleus to begin with, and the effects of flow on its localization appear minor, given the scale of the y-axis.
7. siRNA analysis in HUVECs. Only one siRNA is used (or is it a pool), and the sequence(s) are not provided. All experiments must be carried out with at least two and preferably three independent siRNAs, to reduce the possibility that results are due to an off-target effect. The sequences must be provided for siRNAs.
8. GSK3β data: The description in the text of links between aPKC and GSK3β only quotes one of several papers that have researched this area. For example, it has been reported that GSK3β is a substrate for aPKC, and this is not mentioned in the text.
9. Quantification of western blots from multiple independent experiments is required to show that results are reproducible (e.g. Fig. 4). What happens at different flow rates (Fig. 4a)?
10. Figure 4: PLA should be used for endogenous proteins, not overexpressed proteins (for which FRET would be a better method to test for interaction).

Main changes needed to text/figures (this list is not exhaustive):
1. What is isolectin-B4 and what does it stain for? It is not mentioned in the text.
2. Explain what staining for Erg is used for and why.
3. Extended Fig. 1a, it would help to what is proximal and distal on the figure, as well as where the 'descending aorta' was analysed or any other regions referred to in any other figures (e.g. Fig. S2). It is easier to follow the workflow if the information on different aortic regions is all in one diagram.
4. Extended Fig. 3: This should include body weight of mice, according to the figure legend, but the data are missing.
5. Extended Fig. 2a: in what aged animals was this analysis done? Figure legend states P56, but it should be the same timepoint as Figure 2?
6. Methods - there is a reference missing on how the aortic ECs were isolated.
7. Extended Fig. 4a: at what time point after flow onset were the images shown here taken? Methods and figure panel disagree about the Golgi antibody used.

Referee #3:

In Hikita et al, "PAR-3 controls endothelial planar polarity and vascular inflammation under laminar flow", the authors utilize an inducible conditional knockout of the polarity gene Pard3 encoding PAR-3 specifically in endothelial cells using the Cadherin-5 (Cdh5-CreERT2) Cre driver + tamoxifen to focus specifically on the contribution of PAR-3 to endothelial cell polarity in response to low or moderate blood flow. The authors combine in vivo analysis of PAR-3 with in vitro knockdown studies with siRNA & overexpression studies to address how asymmetry of PAR-3 is

controlled by flow, and how GSK-3 beta and acetylated tubulin (stabilized microtubules) are affected in PAR-3 mutants/PAR-3 siRNA cells: GSK-3 beta is hyperactivated (less Ser9 phosphorylation), and microtubules are destabilized. The authors demonstrate by FRET analysis that PAR-3 co-localizes with aPKC lambda at the front edge of a cell, allowing GSK-3 beta to be phosphorylated on Ser9 to inactivate it, and dissociates from aPKC lambda at the rear edge of the cell, where GSK-3 beta is active and bound to aPKC lambda. Further, the authors show that flow-activated Rho signaling is essential for the asymmetric distribution of PAR-3/aPKC lambda and GSK-3 beta/aPKC lambda: inhibiting Rho kinase leads to increased aPKC/PAR-3 association.
In addition to its role in polarity, the authors dissect the inflammatory response from the planar cell polarity status of the ECs in Pard3 conditional knockouts by addition of GSK-3 beta inhibitor: the inhibitor is only able to block the nuclear p65 accumulation/inflammatory response, but the cell polarity is still compromised in Pard3 mutants. Thus, PAR-3 is critical for cell polarity but not for the pro-inflammatory response in ECs.

Major Problems
Problem 1: the authors state "Importantly, pharmacological suppression of GSK3β restored increased NF-κB nuclear localization but not endothelial PCP. Our results indicate an unexpected relationship between endothelial PCP and vascular inflammation downstream of PAR-3."
This separation lessens the therapeutic importance of their findings, but provides other new avenues to pursue. Regardless, Pard3 is still a likely genetic contributor to genetic predisposition to atherosclerosis and inflammation, which should not be overlooked. The fact that GSK3 beta blockade can reduce the inflammation caused by loss of PAR3 only points to the fact that they share a common pathway, and that GSK3 attenuation could reduce athero risk in patients with Pard3 mutations. It does not lessen the importance of their study that can dissociate the inflammation from endothelial polarity, which is an interesting finding and novel. The authors need to discuss this to greater extent in their discussion.

Problem 2: "These results suggest that PAR-3 is important for endothelial PCP in vivo at low-to-moderate but not at high levels of shear stress."
Unfortunately the only place with dyn/cm2 is extended fig 4, an experiment performed with mouse aortic EC at 12, 18 and 30 dyn/cm2 laminar flow. Although every cell type is different; for HUVEC & BAEC have responses of low-medium-high in the 0-4, 4-12, >12 range. In contrast, Mouse EC are exposed to much higher shear stress in vivo; in the proximal aorta probably averages 60 dyn/cm2 and distal 20-40 dyn/cm2. Text says: "Golgi polarization was compromised in PAR-3 knock down (KD) cells in the presence of low-to-moderate flow but not when exposed to high flow (Extended Fig. 4a-c)." The authors need to determine the appropriate levels of shear stress for their mouse endothelial cells based on cell polarization, expression of VCAM-1, and intracellular signaling mechanisms such as NF-kB. This is especially important because most researchers use Mouse EC from pulmonary blood vessels.

Problem 3: I am not sure that the conclusions regarding RhoA-GSK data are rigorous. The authors state: "Shear stress controls the spatio-temporal antagonism of the PAR-3/aPKClambda/iota (aPKCλ), one of two isoforms of aPKC, complex versus the GSK3β/aPKCλ complex through the RhoA/Rho-kinase pathway, resulting in spatially controlled microtubules stabilization in the axis of flow."
The data which directly support RhoA and GSK-PAR3 are 1) Fig. 4c: Rho kinase inhibitors in 293 cells transfected w/ tagged PAR3, GSK3beta and aPKC lambda, 2) Fig. 4d/e: FRET localization in HUVECs + moderate flow, transfected w/ RhoA-biosensor, showing RhoA is enriched in the rear, in contrast to low flow where RhoA was not asymmetrically localized; and 3) Ext Fig. 6c: These data should be combined w/ Fig. 4c for clarity and direct comparison. Rho kinase inhibitor applied to 293 cells expressing aPKC, GSK3, and PAR3 results in 50% increase in co-IP of PAR3 and aPKC lambda. Blocking Rho signaling increases binding of PAR3 and aPKC, but decreases GSK3-aPKC binding. These data show 1) the effect of Rho signaling and flow rate on Rho localization, and 2) that blockade of Rho kinase tilts the aPKC -GSK3 -PAR3 axis toward aPKC-PAR3 binding, and away from aPKC-GSK3 binding.
The authors make the assumption that flow equates to Rho signaling, but testing their hypothesis directly would help solidify their case. For example, it would be more convincing to see proximity ligation assay (PLA) data with Rho kinase inhibitor showing that asymmetric localization of the GSK3-aPKC and PAR3-aPKC complexes is lost in the absence of Rho activation.

Major Comments
1. I do not see Figure 1 as essential to the paper, and I think it confuses the story. I would remove it. If they leave it in, the authors should at least discuss the lack of obvious apical/basal cell polarity defects in the retinal vasculature in the context of disrupted Golgi orientation and loss of acetylated tubulin that they find in Pard3 cKO ECs.
2. The authors chose to exclude several figures from the main manuscript, which add significantly to the strength of the manuscript data (eg. Extended figs. 2,3 (with revision listed below),4, and 9); I strongly advise them to alter the existing figures to include these data if possible (see below).
3. I would like to see more of a discussion of the findings in the current study with other publications in the field. For example, the authors fail to mention the Colosimo paper from 2010 in which they show in Drosophila epithelial cells that GSK-3 beta regulates aPKC levels by direct phosphorylation.
4. The authors should discuss the physiological significance of Golgi localization and speculate as to why it is so sensitive to cell polarity; including this would emphasize the importance of their work and increase the significance of their findings.

Minor points to be addressed:
1. Extended Figure 1A: There is a misspelling of marginal as "merginal"; please fix. This panel can be removed; only 1B is needed.
2. Figure 2A: Why does the GM130/Golph staining look extracellular? It should be intracellular (Golgi).
3. Figure 2A: What is ERG? I am unfamiliar with this antigen, and do not see any explanation of the antigen in the legends, methods section, or in the results.
4. Figure 2D: What do the dashed vertical lines on the graph represent? Please explain in the legend and/or text.
5. Figure 3A: These en face micrographs show beautiful staining of the aorta, but I'm not sure that they support the authors' argument that EC polarity is defective in Pard3 cKO; the images look highly similar.
6. Figure 3B: The p65 immunostaining is very weak and unconvincing; perhaps the authors should use a phosphorylated anti-p65 antibody, or a nuclear counterstain?
7. Figure 3B: Why does the inner curvature anti-VE cadherin staining look worse than in the Figure 3A micrographs? And why does the anti-Golph staining appear to be decreased in the Pard3 cKOs? Is there in fact less overall staining in the Pard3 ECs?
8. Extended Figure 2A: The anti-VCAM staining data is striking; why is this data not in the main body of the manuscript?
9. Extended Figure 2B: How is the VCAM-1 volume data collected? This measurement is not standard and a bit strange. And why is the number of positive cells not reported instead?
10. Figure 3: In the results section, the authors state that body weight is not affected in the Pard3 cKO mice. Please include a graph or table of the body weight data, or include in the results text; it is not clear whether only the Pard3 mutants showed no weight gain on the high-fat diet, or whether neither group gained weight.
11. Extended Figure 3: It is difficult to determine where the MOMA-2-positive cells are located relative to the aortic arch; please counterstain the tissue, or show a low-power image for orientation purposes.
12. Extended Figure 3: This is an important data figure for both the assessment of NF-kB activation and atherogenesis, and should be included in the manuscript proper. Along with this data figure, please include quantification of the increased staining in Pard3 cKO vessels.
13. Figure 4: Why is the rate of flow not given for this figure? In extended Figure 4, a great deal of emphasis was placed on the specific strength of flow; it is strange that the authors then simply state "the effect of flow" in Figure 4, rather than state how much flow. And are the cells used in Figure 4 HUVEC ECs? Please state the cell type in the results section.
14. Extended Figure 6A: The authors state that there are increased amounts of acetylated tubulin in 6BIO (GSK3beta inhibitor)-treated cells; however, the western blot is not convincing. Please show quantitative data to support the increase observed.
15. Figure 5: Please correct the spelling mistake "Sildewall" to sidewall.
16. Figure 5A: Why are levels of VE-cadherin staining reduced in the Pard3 cKO vessels? If they are not reduced, please find alternative images that are more representative.
17. Figures 5B, 5C: Please label the Y axes on the graphs. For each graph, I would remove the inner and sidewall data points, since marginal ECs are the focus of the figure. Also, please change the figure legend which states that 5B is Golgi orientation, and 5C is percentage nuclear p65 (reversed).

18. Extended Figure 6C: I don't see what the authors are trying to point out.
19. In Extended Figure 6D, is PLA the lentiviral GFP-PAR3-aPKC lambda? Please indicate clearly.
20. Extended Figure 9: This is a helpful model figure and should be included in the manuscript figures.

1st Revision - authors' response 30 January 2017

Response to reviewers (EMBOR-2017-45253V1)

We would like to thank all the reviewers for their very high evaluation for our manuscript. We also appreciate their helpful comments and suggestions that have allowed us to substantially improve the manuscript. As you will see, we have extensively revised the manuscript by adding a large amount of new data. While a detailed point-by-point response to all comments is given further below, here is a brief summary of the most important changes in our revised manuscript.

1) According to the reviewers' suggestions, we reorganized the figures. As a result, we now present our manuscript as an article style. Additionally, we expanded the discussion section as well as the results section, which allows us to better organize the figures and present data.

2) For easier understanding by the readers, we changed the style of data presentation showing the relationship between PAR-3 and endothelial cell polarity in the growing retinal vasculature at P6 (Figure1, Figure Extended View1 and Appendix1).

3) To examine the effect of flow on endothelial aPKC/GSK3b and PAR-3/aPKC complex formation, we performed immunoprecipitation of transfected tagged version of these proteins in cultured ECs. The amount of the PAR-3/aPKC complex was increased in the presence of flow, whereas that of aPKC/GSK3b was reduced (Figure 4G). Additionally, we examined the spatial distribution of endogenous protein complexes in cultured ECs with PLA (Figure 5C, D)

4) To eliminate the potential off-targeting effect of siRNA mediated PAR-3 KD in cultured ECs, we used a second siRNA sequence in key experiments and reproducibility was confirmed (Figure Extended View 3 and Appendix 3).

5) To further confirm the effect of flow on ECs, we examined Golgi localization and VCAM-1 expression using ECs isolated from mice aorta. Arterial ECs needed to be exposed to higher shear stress (30 dyn/cm^2) to be polarized, and we confirmed that loss of PAR-3 resulted in mispolarization. Shear stress of 18 dyn/cm^2 was not enough for EC polarization torward the axis of flow in 120 min. Also VCAM-1 transcription was reduced in the presence of flow in ECs isolated from control mice but not from PAR-3 KO mice (Appendix 2).

6) For better data presentation and to avoid misunderstanding, we replaced some images and panels of western blotting, according to the reviewers' suggestions. All of the western blots are now presented with quantification and statistics.

7) Now we provide anti-MOMA2 antibody staining in en face aorta staining with quantification and statistical analysis.

Thus, the sum of all the data in the manuscript strongly supports that PAR-3 controls endothelial polarity to the flow axis but is not essential for apical basal polarization in a manner dependent on shear rate. These compelling findings establish a new understanding and conceptual advancement of the role of the cell polarity complex in endothelial health and disease. Given the fact that GSK3b is involved in many serious diseases, such as inflammation, Alzheimer's disease, diabetes, cancer and bipolar disorder, our findings may open potential new strategies for the treatment of multiple pathogeneses.

Referee#1:

1. Fig 1 does not convincingly show normal apical-basal polarity. 3D sections that actually reveal polarity are required to make this point.
Reply
According to the reviewer's suggestion, now we included the 3D reconstituted images in the figure (Figure Extended View 1D). Also, we moved this figure into the Extended View to better focus on the role of PAR-3 in endothelial polarity toward flow, according to the other reviewers' suggestion.

2. Contrary to the text, fig 2b shows no failure to orient in flow in arteries or capillaries, and only a slight effect in veins. There is also slight effect at the sprouting front, despite the claim that it is unaffected. Fig 2D explains this better as a shift in sensitivity but the initial presentation of the data is problematic since the text does not match the data.
Reply
I am sorry for causing this misunderstanding. This is due to the poor quality of the data presentation, as the reviewer's suggested. Now we have improved the data presentation in Figure 1 and Appendix 1. We think this change improves the quality of the manuscript.

3. The increased NF-kB in the aorta from Par3-/- mice does not correlate with Golgi orientation. There is an increase even in the inner curvature region where orientation/alignment do not differ. So par3 effects on these variables appear to be independent. They eventually say something about this in the Discussion but the way the data are presented and analyzed leads one to believe that these 2 effects should be linked, which contributes to the confusion. For example, they conclude that "Thus, PAR-3 inhibits atherosclerosis onset by promoting endothelial PCP and blocking endothelial inflammation", which is not supported by the data.
Reply
Thank you for raising this important point. PAR-3 is important for GSK3b activation and endothelial planar cell polarization under flow. While spatial antagonism of PAR-3/aPKC with aPKC/GSK3b complexes is essential for PCP formation, spatial distribution of the complexes is not necessary for an inflammatory response downstream of GSK3b. To avoid confusion, first, we included model figures within the main figures (Figure 7). Moreover, we improved the discussion section to clearly describe this conclusion, p15-16, line 349-401.

4. Results in extended fig 3 need to be quantified. All of the Western blots throughout the paper need to be quantified from multiple experiments and statistics analyzed.
Reply
Accordingly, we quantified en face aorta MOMA-2 staining and presented these data in Figure 3E and 3F. Also, we included quantified values of western blotting from 3 independent experiments with statistical analysis in each figure (Figure 4A, B, D, E, F, and G).

5. Plasma lipids need to be determined in WT and par3 iec apoE-/- mice on high fat diets.
Reply
According the reviewer's request, we measured plasma lipid values and included these data in Figure 3C. The level of serum cholesterol in ApoE KO mice and ApoE/PAR-3 KO mice was equivalent.

6. The experiments with co-IP of GSK3b, Par3 and aPKC are the most mechanistic ones in the paper but effects of flow on these complexes was not analyzed. This is an important point that would link several of the observations in the study.
Reply
According to the reviewer's request, we carried out immunoprecipitation of GSK3b, PAR-3 and aPKC under flow and included this result in Figure 4G. Flow induced PAR-3/aPKC complex formation and reduced aPKC/GSK3b complex formation. This is consistent with the previous report that total cellular RhoA activity in ECs is reduced under flow (Tzima et al EMBO J 2002). On the other hand, local RhoA activity at the rear edge of ECs was increased (Figure 5A, B), which facilitates the distinct localization of the polarity complexes in ECs (Figure 5C and D, Figure Extended View 4B-E).

7. The NF-kB results in extended fig 5 are puzzling. They show that flow triggers a rapid decrease in nuclear p65 instead of an increase, as has been reported in many papers and also in Extended fig 8. The decrease is merely less after Par3 knockdown,
Reply
I agree with this point. As reviewer #3 mentioned in his comment "Problem2", the responses of different types of ECs such as HUVEC and aorta endothelial cells to flow could be different. This might be the cause of this puzzling result with HUVEC. Since we completed the same experiment with isolated arterial ECs from both the control and PAR-3 KO mice to support in vivo finding (Figure Extended View 5, Figure 6), we decided to take out this old Extended Figure 5. Additionally, we confirmed that the role of PAR-3 as a sensitizer of endothelial Golgi orientation toward flow in isolated arterial ECs from mice. As the number of isolated ECs from mice is limited, and they are not transfectable, we could not repeat all of the experiments with isolated ECs from mice. However, the rescue experiments using GSK3b inhibitor with mice strongly support our conclusion.

8. The experiments showing differential associations of Par3, aPKC and GSK3b in 293 cells needs to be confirmed in endothelial cells. In this regard, it is puzzling that extended fig 6d,e analyzes a complex of GSK3 with VE-cadherin instead of with aPKC, which would correlate with Fig 4c.
Reply
Sorry for causing this misunderstanding. The original figure showed GSK3b/aPKC complex localized at the EC-EC junction region. We repeat the PLA experiments to show the distinct localization of the GSK3b/aPKC and PAR-3/aPKC complexes in cultured EC using endogenous proteins in Figure 5C, D. Additionally, we carried out immunoprecipitation of GSK3b, aPKC and PAR-3 using cultured ECs under flow (Figure 4G).

9. Fig 5b. The quantification seems to show that in Par3KO cells, BIO increases golgi orientation in the marginal zone and sidewall. Is this effect significant?
Fig 5c is confusing. How can nuclear p65 be negative? Further, it shows nuclear p65 elevation in the sidewall instead of the inner curvature. Something must be mislabeled.
In any case, it would be helpful to indicate what is quantified on the Y axis in the figure.
Reply
Thank you for this comment and sorry for causing additional confusion. We included the labeling of the Y axis to avoid misunderstanding (Figure 6B, C).

10. The idea that loss of cell polarity in ECs leads to pathology is exactly opposite to the data showing that effects of Par3 and GSK3 on polarity and NF-kB are separable.
Reply
I am sorry for causing confusion. The idea is that it is not necessary to retain cell polarity to prevent inflammation. To avoid this confusion, first we included model figures within the main figures (Figure 7). Moreover, we improved the discussion section to clearly describe p15-16, line 349-401..

Minor Points

Planar cell polarity generally refers to non-canonical Wnt signaling, which has not been shown to mediate endothelial cell alignment or orientation in flow. Further, while alignment has been linked to inflammation (ref 40), golgi orientation (which is closer to traditional PCP) has not. Indeed, the cited references in the Introduction (5 and 6) never use the term PCP in discussing effects of flow on endothelial cells. The authors need to clarify these points and to be more careful about using these terms correctly.
Reply
According to the suggestion, we described "planar cell polarity represented by Golgi orientation toward the flow axis" and "endothelial polarity or polarization toward the flow axis" in the whole manuscript.

It is not correct that "the role of PAR-3 in endothelial polarization in vivo has not been shown". See Liu et al J Cell Biol. 2013 Jun 10;201(6):863-73. Polarized formation of a number of protein complexes requires Par3.
Reply

Thank you for pointing this out. Now we describe "the role of PAR-3 in endothelial polarization in living organisms has not been shown" in P4, line 85-56.

p. 5. VE-cadherin forms a homophilic complex, not a hemophilic complex.
Reply
Thank you for pointing out our mistake. Accordingly we corrected our description.

Fig 4a. previous work has reported flow induces phosphorylation of GSK3b on Ser9, which was linked to tubulin stability. See McCue Circ Res. 2006 Apr 14;98(7):939-46. And Biswas Am J Pathol. 2006 Jul;169(1):314-24..
Reply
Thank you for the comment. We citied this manuscript at P10, line 237.

It would be helpful to put extended fig 6b and fig 4c next to each other since they are complementary. Similarly, Fig 4f,g and extended 6d,e should be shown together.
Reply
We now increased the number of figures, thus, we are able to present the data as suggested. Please see the new Figure 4.

The authors make the point in their Discussion that "Unlike in epithelial cells, endothelial PAR-3 did not seem to be critical for the establishment of apical basal polarity and cell-cell junction formation". Actually, inhibition of par3 in epithelial cells generally gives rise to a partial defect in apical-basal polarity, slowing cell polarization but not necessarily blocking at later times. This may not be so different from the shift in sensitivity to flow magnitude reported here.
Reply
Yes, we agree with your comment. We took out the description "Unlike in epithelial cells" from this sentence.

Referee #2:

Other major points:
1. How efficient is PAR3 depletion in mouse endothelial cells following tamoxifen administration? It is essential to quantify this because inducible depletion can be very variable between models.
Reply
Please see my previous publications (Wang et al., Nature 2010; Nakayama et al., 2013, Nat Cell Biol). In the paper in 2010, we generated VE-cadherin-CreERT2 mice line. There we observed the cre-recombination efficiency (Sup. Fig. 3C, Wang et al., Nature 2010) and confirmed the cre activity in almost all endothelial cells. In the paper in 2013, we did immunostaining of PAR-3 in control and PAR-3 KO mice (Figure 5B Nakayama et al., Nat Cell Biol 2013). While the immunoreactive signal against the PAR-3 antibody was observed at EC-EC junction sites, this signal was diminished in PAR-3 EC specific KO mice. We used exact the same mice line in this study.

2. Figure 1: The sprouting defect of PAR3-depleted retina is not so obvious from the images and needs quantifying to know if it is similar to previous analysis. What is isolectin-B4 and what does it stain for? It is not mentioned in the text.
Reply
According to the reviewer's suggestion, we included the quantification showing the sprouting defect (Figure Extended View 1C). Isolectin-B4 specifically binds terminal a-galactosyl residues expressed by various cells including endothelial cells. Particularly in the retina angiogenesis model, it is a very well established endothelial cell marker. This information is inserted in the methods section with reference information p40, line 938.

3. Figure 2: The full methodology for the quantification of retinal polarity and for estimating flow rates and flow direction needs to be provided, otherwise it is not possible to understand how the results were generated, or justify the conclusion that Par3 is only required at low to medium shear stress in vivo. The work-flow for the quantification should be shown in a supplementary figure, because it is very difficult to follow the method. Enlarged regions of the

vessels should be shown in single colors so that the Golgi localization and how it is scored can be clearly understood. Are the vessels shown capillaries? Images should be expanded to show some of each vessel type and label each vessel type on the images. The effects on polarity of the Golgi appear fairly minor.
Reply
The detail methodology for the quantification of retinal polarity and for estimating flow rates and flow direction is now published (Bernabeu MO et al., 2017 bioRxiv 237602). We citied this paper. According to the reviewer's request, we now changed the way of data presentation and replaced most of the panels, which is easier for the readers to understand. Please see new Figure 1 and Appendix 1.

4. Extended Fig. 1b: the Erg staining does not appear restricted to the nucleus here, so it is difficult to see how the orientation of the Golgi with respect to the nucleus was determined.
Reply
To avoid this confusion, now we replaced the images in the figure, which improved the quality of data (Figure Extended View 2B).

5. Figure 3d/e: quantification of the Oil red area should be done in µm2 rather than pixels, to give the absolute area, and should also take into consideration the total area measured, because the aortas from PAR3-depleted mice could have a different size/area than control mice.
Reply
Thank you for point out this. Accordingly we updated this figure (Figure 3B).

6. Extended Fig. 5: How many separate experiments were analysed for NF-kB nuclear localization? The authors state more than 100 cells were analysed, but in how many independent experiments? What happens at 24 h after flow initiation? Why is the NF-kB nuclear localization high in control cells before the onset of flow? It should be very low, since it is known that flow transiently increases NF-kB nuclear localization from published studies. Are the cells under pro-inflammatory conditions, which would induce its nuclear localization? The text referring to this figure is inaccurate, because NF-KB is in the nucleus to begin with, and the effects of flow on its localization appear minor, given the scale of the y-axis.
Reply
As pointed by other reviewers, this result is puzzling. According to the third reviewer, this would be due to the character of HUVEC. For inflammation studies, arterial derived endothelial cells are often used. Thus, we decided to take out this figure. Instead, we further characterized ECs isolated mice aorta. Consistent with the analysis with HUVEC, PAR-3 was involved in EC Golgi orientation toward flow (Appendix 3B). Flow induced VCAM-1 downregulation was compromised in PAR-3 KO ECs (Appendix 2). Under the conditions, NF-kB nuclear localization was enhanced in PAR-3 KO ECs (Figure EV5). These observations further support our data using the mouse model (Figure 6).

7. siRNA analysis in HUVECs. Only one siRNA is used (or is it a pool), and the sequence(s) are not provided. All experiments must be carried out with at least two and preferably three independent siRNAs, to reduce the possibility that results are due to an off-target effect. The sequences must be provided for siRNAs.
Reply
Thank you for the comment to allowing us to impair the reliability of our data. All of experiments were repeated at least 3 times. Representative images were shown. Additionally, we now included data from experiments conducted with a second siRNA with a different sequence. Importantly, we would like to emphasized that key observations, such as shear-rate dependent Golgi misorientation or increased NFkB nuclear localization, were confirmed with endothelial specific PAR-3 KO mice, suggesting the effect of the siRNA is not due to an off-target effect.

8. GSK3β data: The description in the text of links between aPKC and GSK3β only quotes one of several papers that have researched this area. For example, it has been reported that GSK3β is a substrate for aPKC, and this is not mentioned in the text.
Reply

Accordingly, we citied the manuscript by Colosimo et al., 2009 and discussed the potential relationship of a balance between the aPKC/GSK3b and the PAR-3/aPKC complexes in p17, line 411.

9. Quantification of western blots from multiple independent experiments is required to show that results are reproducible (e.g. Fig. 4). What happens at different flow rates (Fig. 4a)?
Reply
We quantified densitometric values of three independent experiments and included statistic analyses for Figure 4. Regarding the effect of different flow rates on GSK3b phosphorylation, we could not see a significant difference between control and PAR-3 KD ECs at 30 dyn/cm^2 and 12 dyn/cm^2. Please see Appendix 3D, E.

10. Figure 4: PLA should be used for endogenous proteins, not overexpressed proteins (for which FRET would be a better method to test for interaction).
Reply
According to the reviewer's suggestion, we now performed the PLA analysis on endogenous proteins and include data in Figure 5C, D. This new data is, which is consistent with our previous conclusion.

Main changes needed to text/figures (this list is not exhaustive):
1. What is isolectin-B4 and what does it stain for? It is not mentioned in the text.
Reply
Isolectin-B4 specifically binds terminal a-galactosyl residues expressed by various cells including endothelial cells. Particularly in the retina angiogenesis model, it is a very well established endothelial cell marker. This information is inserted in the methods section with reference information p40, line 938.

2. Explain what staining for Erg is used for and why.
Reply
Erg is an established of endothelial nucleus with citation. We added this information in p5, line 108.

3. Extended Fig. 1a, it would help to what is proximal and distal on the figure, as well as where the 'descending aorta' was analysed or any other regions referred to in any other figures (e.g. Fig. S2). It is easier to follow the workflow if the information on different aortic regions is all in one diagram.
Reply
Thank you for the comment. Accordingly, we included this information (Figure Extended View 2A).

4. Extended Fig. 3: This should include body weight of mice, according to the figure legend, but the data are missing.
Reply
Sorry for the confusion. According to the reviewer's suggestion, we included this information in Figure 3D.

5. Extended Fig. 2a: in what aged animals was this analysis done? Figure legend states P56, but it should be the same timepoint as Figure 2?
Reply
Sorry for this confusion. The direct answer is no. The analysis in the retinal vasculature has been done with P6 mice. On the other hand, the experiments using mice aorta was examined at P56.To examine the effect of PAR-3 on atherosclerosis formation, mice were bred on the ApoE-/- background and scarified at 18 weeks old.

6. Methods - there is a reference missing on how the aortic ECs were isolated.
Reply
Thank you for your comment. We now included the reference (ref number 61).

7. Extended Fig. 4a: at what time point after flow onset were the images shown here taken? Methods and figure panel disagree about the Golgi antibody used.

Reply
Sorry for the confusion and thank you for this comment. These pictures were taken after 1hr of flow with a Golph4 antibody. This antibody information is written in the figure (Figure Extended View 3A).

Referee #3:

Major Problems
Problem 1: the authors state "Importantly, pharmacological suppression of GSK3β restored increased NF-κB nuclear localization but not endothelial PCP. Our results indicate an unexpected relationship between endothelial PCP and vascular inflammation downstream of PAR-3."
This separation lessens the therapeutic importance of their findings, but provides other new avenues to pursue. Regardless, Pard3 is still a likely genetic contributor to genetic predisposition to atherosclerosis and inflammation, which should not be overlooked. The fact that GSK3 beta blockade can reduce the inflammation caused by loss of PAR3 only points to the fact that they share a common pathway, and that GSK3 attenuation could reduce athero risk in patients with Pard3 mutations. It does not lessen the importance of their study that can dissociate the inflammation from endothelial polarity, which is an interesting finding and novel. The authors need to discuss this to greater extent in their discussion.
Reply
Thank you for the high evaluation on our findings. According to the suggestion, we expanded the discussion section p15-18. As pointed out by the reviewer, genetic analysis of human patients suffering from atherosclerosis with PAR-3 mutation has not been analyzed yet. This would be interesting and further analysis is warranted. We directly included described this point within the discussion section.

Problem 2: "These results suggest that PAR-3 is important for endothelial PCP in vivo at low-to-moderate but not at high levels of shear stress."
Unfortunately the only place with dyn/cm2 is extended fig 4, an experiment performed with mouse aortic EC at 12, 18 and 30 dyn/cm2 laminar flow. Although every cell type is different; for HUVEC & BAEC have responses of low-medium-high in the 0-4, 4-12, >12 range. In contrast, Mouse EC are exposed to much higher shear stress in vivo; in the proximal aorta probably averages 60 dyn/cm2 and distal 20-40 dyn/cm2. Text says: "Golgi polarization was compromised in PAR-3 knock down (KD) cells in the presence of low-to-moderate flow but not when exposed to high flow (Extended Fig. 4a-c)." The authors need to determine the appropriate levels of shear stress for their mouse endothelial cells based on cell polarization, expression of VCAM-1, and intracellular signaling mechanisms such as NF-kB. This is especially important because most researchers use Mouse EC from pulmonary blood vessels.
Reply
To adders this point, we isolated ECs from control and KO mice aorta. These ECs were subjected to different rates of flow we and examined Golgi polarization and VCAM-1 expression. As pointed out by the reviewer, 18 dyn/cm^2 was not enough to induce cell polarization. However, when ECs were subjected to 30 dyn/cm^2 flow, we could observed Golgi polarization in ECs from control mice but not from PAR-3 KO mice, suggesting the role of endothelial PAR-3 as a sensitizer toward flow (Appendix 3B). VCAM-1 transcription was reduced in the presence of flow in control ECs, which was compromised in PAR-3 KO ECs (Appendix 2).

Problem 3: I am not sure that the conclusions regarding RhoA-GSK data are rigorous. The authors state: "Shear stress controls the spatio-temporal antagonism of the PAR-3/aPKClambda/iota (aPKCλ), one of two isoforms of aPKC, complex versus the GSK3β/aPKCλ complex through the RhoA/Rho-kinase pathway, resulting in spatially controlled microtubules stabilization in the axis of flow."
The data which directly support RhoA and GSK-PAR3 are 1) Fig. 4c: Rho kinase inhibitors in 293 cells transfected w/ tagged PAR3, GSK3beta and aPKC lambda, 2) Fig. 4d/e: FRET localization in HUVECs + moderate flow, transfected w/ RhoA-biosensor, showing RhoA is enriched in the rear, in contrast to low flow where RhoA was not asymmetrically localized; and 3) Ext Fig. 6c: These data should be combined w/ Fig. 4c for clarity and direct comparison. Rho kinase inhibitor applied to 293 cells expressing aPKC, GSK3, and PAR3

results in 50% increase in co-IP of PAR3 and aPKC lambda. Blocking Rho signaling increases binding of PAR3 and aPKC, but decreases GSK3-aPKC binding. These data show 1) the effect of Rho signaling and flow rate on Rho localization, and 2) that blockade of Rho kinase tilts the aPKC -GSK3 -PAR3 axis toward aPKC-PAR3 binding, and away from aPKC-GSK3 binding. The authors make the assumption that flow equates to Rho signaling, but testing their hypothesis directly would help solidify their case. For example, it would be more convincing to see proximity ligation assay (PLA) data with Rho kinase inhibitor showing that asymmetric localization of the GSK3-aPKC and PAR3-aPKC complexes is lost in the absence of Rho activation.

Reply

Thank you for this comment to improve the reliability of our data. We examined immunoprecipitation of GSK3b, PAR-3 and aPKC under flow and included this result in Figure 4G. Flow induced PAR-3/aPKC complex formation and reduced aPKC/GSK3b complex formation. This is consistent with the previous report that total cellular RhoA activity in ECs is reduced under flow. According to the reviewer's suggestion, we repeated the PLA assay with endogenous proteins in the presence and absence of Rho-kinase inhibitor (Figure 5C, D). Local RhoA activity at the rear edge of ECs was increased (Figure 5A, B), which causes asymmetric localization of the polarity complexes in ECs (Figure 5C and D, Figure Extended View 4B-E). Treatment with Rho-kinase inhibitor induced aPKC/PAR-3 complex formation that was confirmed with immunoprecipitation using cultured ECs (Figure 4F) and PLA (Figure 5C, D).

Major Comments

1. I do not see Figure 1 as essential to the paper, and I think it confuses the story. I would remove it. If they leave it in, the authors should at least discuss the lack of obvious apical/basal cell polarity defects in the retinal vasculature in the context of disrupted Golgi orientation and loss of acetylated tubulin that they find in Pard3 cKO ECs.

Reply

According to the reviewers' suggestion, first of all, we put these data into Figure Extended View 1 to better focus on the endothelial PCP. Additionally, we included 3D reconstituted images (Figure Extended View 1D).

2. The authors chose to exclude several figures from the main manuscript, which add significantly to the strength of the manuscript data (eg. Extended figs. 2,3 (with revision listed below),4, and 9); I strongly advise them to alter the existing figures to include these data if possible (see below).

Reply

Thank you for your positive evaluation for our experiments. Now we changed the format of our manuscript from letter to article style, which allows us to leave most of the data in the main figures.

3. I would like to see more of a discussion of the findings in the current study with other publications in the field. For example, the authors fail to mention the Colosimo paper from 2010 in which they show in Drosophila epithelial cells that GSK-3 beta regulates aPKC levels by direct phosphorylation.

Reply

Yes, we included this information at p17, line 411. Colosimo et al., has shown that GSK3ß directly phosphorylates aPKC inducing ubiquitin dependent protein degradation, suggesting GSK3ß is both upstream and downstream of aPKC. The balance between the aPKC/GSK3ß and the aPKC/PAR-3 complexes might be involved in positive or negative feedback signaling of GSK3ß, therefore acting as a signaling hub for cellular responses.

4. The authors should discuss the physiological significance of Golgi localization and speculate as to why it is so sensitive to cell polarity; including this would emphasize the importance of their work and increase the significance of their findings.

Reply

Golgi orientation represents not the only direction of cytoskeletal networks within the cells but also the direction of intracellular transport. Many secreted factors from ECs would be important for inflammation, thus we thought misoriented Golgi in the tissue could be directly upsteam of inflammation, which was obviously not the case in our study. Cytoskeletal reorganization is a dynamic process, which might be important for motile behavior of cells. Consistently, vessel pruning, which is achieved by directed cell migration (Franco et al., Plos Bio 2015), was increased

in P6 retinal vasculature of EC specific PAR-3 KO mice (Nakayama et al., NCB 2013). This would be due to the defected Golgi orientation in ECs. We discussed this point in p16-17 line 397-401.

Minor points to be addressed:
1. Extended Figure 1A: There is a misspelling of marginal as "merginal"; please fix. This panel can be removed; only 1B is needed.
Reply
Thank for raising this point to improve our manuscript. We corrected this information.

2. Figure 2A: Why does the GM130/Golph staining look extracellular? It should be intracellular (Golgi).
Reply
There are other cell types such as pericytes and gilal cells, which are also GM130/Golph4 positive. To avoid misunderstanding, we replaced most of the panels in this figure to improve the quality of the data.

3. Figure 2A: What is ERG? I am unfamiliar with this antigen, and do not see any explanation of the antigen in the legends, methods section, or in the results.
Reply
Erg is an established of endothelial nuclei. We added this information in p5, line108.

4. Figure 2D: What do the dashed vertical lines on the graph represent? Please explain in the legend and/or text.
Reply
To avoid misunderstanding, we took out these lines (Figure 1D).

5. Figure 3A: These en face micrographs show beautiful staining of the aorta, but I'm not sure that they support the authors' argument that EC polarity is defective in Pard3 cKO; the images look highly similar.
Reply
To avoid confusion, we replaced these images (Figure 2A).

6. Figure 3B: The p65 immunostaining is very weak and unconvincing; perhaps the authors should use a phosphorylated anti-p65 antibody, or a nuclear counterstain?
Reply
To avoid misunderstanding, we replaced with better images (Figure 2B).

7. Figure 3B: Why does the inner curvature anti-VE cadherin staining look worse than in the Figure 3A micrographs? And why does the anti-Golph staining appear to be decreased in the Pard3 cKOs? Is there in fact less overall staining in the Pard3 ECs?
Reply
To avoid misunderstanding, we replaced them with better images (Figure 2B).

8. Extended Figure 2A: The anti-VCAM staining data is striking; why is this data not in the main body of the manuscript?
Reply
Thank you for the positive evaluation on our data. Now we include this data in the main figures (Figure 2D, E).

9. Extended Figure 2B: How is the VCAM-1 volume data collected? This measurement is not standard and a bit strange. And why is the number of positive cells not reported instead?
Reply
The image presented was the 3D reconstituted images of Z-stacks. Originally, we measured voxel volume and voxel intensity of VCAM-1 signal in single animals by the imaging software, Volocity. The number of positive cells was not countable as we did not double stained with VE-cadherin. To address the comment, we measured intensity of immunoreactive signal of VCAM-1 in the region of the inner curvature from 3 independent animals, then the average intensity was compared between control and KO animals (Figure 2D, E).

10. Figure 3: In the results section, the authors state that body weight is not affected in the

Pard3 cKO mice. Please include a graph or table of the body weight data, or include in the results text; it is not clear whether only the Pard3 mutants showed no weight gain on the high-fat diet, or whether neither group gained weight.
Reply
Thank you for pointing out our mistake in the original version. The graph of mouse body weight is shown in Figure 3D.

11. Extended Figure 3: It is difficult to determine where the MOMA-2-positive cells are located relative to the aortic arch; please counterstain the tissue, or show a low-power image for orientation purposes.
12. Extended Figure 3: This is an important data figure for both the assessment of NF-kB activation and atherogenesis, and should be included in the manuscript proper. Along with this data figure, please include quantification of the increased staining in Pard3 cKO vessels.
Reply
According to the suggestion, we showed en face staining with anti-MOMA-2 antibody. Please see Figure 3E, F.

13. Figure 4: Why is the rate of flow not given for this figure? In extended Figure 4, a great deal of emphasis was placed on the specific strength of flow; it is strange that the authors then simply state "the effect of flow" in Figure 4, rather than state how much flow. And are the cells used in Figure 4 HUVEC ECs? Please state the cell type in the results section.
Reply
In the original Figure4, which is now divided into Figure 4 and Figure 5 in the revised manuscript, HUVECs were subjected 18 dyn/cm^2 flow.

14. Extended Figure 6A: The authors state that there are increased amounts of acetylated tubulin in 6BIO (GSK3beta inhibitor)-treated cells; however, the western blot is not convincing. Please show quantitative data to support the increase observed.
Reply
Accordingly, we quantified the level of acetylated tubulin. Please see Figure Extended View 3.

15. Figure 5: Please correct the spelling mistake "Sildewall" to sidewall.
Reply
Thank you for pointing out our mistake. We corrected this in the revised manuscript (Figure 6).

16. Figure 5A: Why are levels of VE-cadherin staining reduced in the Pard3 cKO vessels? If they are not reduced, please find alternative images that are more representative.
Reply
Accordingly, we replaced images in Figure 6, which was Figure 5 in the original version.

17. Figures 5B, 5C: Please label the Y axes on the graphs. For each graph, I would remove the inner and sidewall data points, since marginal ECs are the focus of the figure. Also, please change the figure legend which states that 5B is Golgi orientation, and 5C is percentage nuclear p65 (reversed).
Reply
Accordingly, we added labeling of the Y axis in Figure 6, which was Figure 5 in the original version.

18. Extended Figure 6C: I don't see what the authors are trying to point out.
Reply
We would like to explain that low level of shear stress failed to induce spatially restricted RhoA activation in cultured ECs.

19. In Extended Figure 6D, is PLA the lentiviral GFP-PAR3-aPKC lambda? Please indicate clearly.
Reply
Sorry for causing confusion. This is PLA of myc-aPKC/GFP-GSK3b transfected into HUVEC.

20. Extended Figure 9: This is a helpful model figure and should be included in the manuscript figures.

Reply
Thank you for the positive evaluation. We reformatted our manuscript from letter to article style and included this model figure into the manuscript figures (Figure 7).

2nd Editorial Decision 13 March 2018

Thank you for the submission of your revised manuscript to our journal.
We have meanwhile received a complete set of reviews from all referees, which I include below for your information.

I apologize again for the delay in handling your manuscript, but as you will see, the referee's opinion remained divided after the revision. All referees have a number of concerns that need to be addressed in a final revision. Referee 1 points out that the conclusion that aPKC triggers dephosphorylation of GSK3b conflicts with published data. Moreover, this referee remarks that the effect of the Rho kinase inhibitor on the localization of the aPKC/GSK3 complex has not been analyzed experimentally and such an analysis should be provided to support the conclusions made. Referee 2 asks for clarification of text, methods, figures and statistics, which should be provided. See also my comments below regarding statistics on single representative experiments. Referee 3 raises a number of concerns and did not support publication of the manuscript in EMBO reports. Upon further discussion with the other referees, we suggest to address point 2, 3, and 4 of referee 3 in the text. Regarding point 3, we note that the HFD was applied for a rather short period of 10 weeks, which can explain the low Oil Red O staining. However, this could be mentioned in the text or figure legend and higher resolution images for the MOMA staining should be provided.

From the editorial side, there are also a few things that we need before we can proceed with the final acceptance of your study:

- You have submitted your article as Scientific Report. Please note that this format is limited to a maximum of five figures. Since your article contains seven figures, I suggest to resubmit it as Article. In this case, also the Results and Discussion section can stay separate.

- Please note that all materials and methods should be part of the main manuscript file in a section called Materials and Methods that follows the Discussion paragraph.

- Please provide the Appendix as a single pdf that contains the title and a table of content with page numbers on the first page and also includes all figure legends.

- Please follow the nomenclature Appendix Figure Sx in the main text and in the figure labels.

- Please supply a running title (max 40 characters incl. spaces)
- Please supply up to five keywords

- Please provide the Author contributions and Conflict of interest as two different paragraphs (in this order).

- Please reformat the references according to journal style, i.e., 'et al' should be used if there are more than 10 authors and the first 10 authors should be listed. You can download the respective Endnote file from our online author guidelines
(https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view)

- Please upload the movies as ZIP file. The ZIP file should contain the movie file and a separate plain text README file with item title and description.

- Please differentiate the two authors with the initials TH in the Author contribution paragraph. I noticed that the author Mengnan Li is listed as LM instead of ML.

- Please provide scale bars for all images. This information is currently missing for Fig. 1A (right panel showing artery, vein, capillary), 2A, 2D (zoom), 3A, 5A, 5C, EV1D, EV2B, C, EV4A, D, and

Appendix Fig. S3B.

- Figures 5D, F and 6B, C: these panels show the quantification of data from one representative experiment. Please note that the application of statistical tests is not appropriate in this case since n = 1. Please re-calculate the mean, SEM and p-value using all the data from the three independent experiments.

- Legend figure 3: it appears that the description of panel C and E are mixed up in the legend.

- I have reviewed the abstract and took to the liberty to make some suggestions. Please review the attached Word document and implement as appropriate.

- 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 have drastically improved the manuscript, which now makes a lot more sense. In general, they have made a good faith effort to unravel a system that is rather complicated. While it isn't perfect, the work is generally done to a high standard. If the can resolve the remaining issues, it would be well worth publishing.

Major criticisms:

The results on aPKC-GSK3b are confusing. They cite literature that,"Previous reports suggest that aPKC, a member of the PAR polarity complex, forms a complex with GSK3ß and controls its activity by phosphorylating Ser9 residue". But their model is that aPKC triggers dephosphorylation of GSK3b, the opposite effect. This needs to be clarified, including some comment about how a kinase mediates dephosphorylation.

Fig 5 and EV4. The effect of the Rho kinase inhibitor is analyzed only for the par3-aPKC complex localization to the front. Yet the authors conclude that "that flow-dependent RhoA/Rho kinase activity determines the spatial distribution of PAR-3/aPKCλand aPKCλ/ GSK3β complexes, which in turn delimits GSK3β activity and microtubule stability". To draw that conclusion, it is necessary to check the effect of Rho kinase inhibition on polarization of the aPKC/GSK3 complex and ac-tubulin, which they have not done.

Minor
Intro: it is unclear what they mean by, "Microtubule organization centers (MTOCs) and the Golgi apparatus are aligned parallel to the flow direction". The MTOC and Golgi are usually considered to be in front or behind the nucleus. Parallel and perpendicular don't really apply.

Referee #2:

The manuscript is much improved through the authors' additions of new data and new analysis of data, as well as revision of the text and figures to make the methods and analysis clearer.
The remaining points need to be addressed by revisions to the text and figures:

1. My original point 1: add reference(s) and text to the methods section on mice to state that tamoxifen efficiently knocks out PAR3, as previously shown.
2. My original point 6: for all graphs showing analysis of cells. The authors still have not stated in most of the figure legends whether the graphs show analysis of cells from just one experiment (in which case statistical analysis is not possible), or whether the analysis is of cells from 3 independent experiments (which have been pooled to give the total number of cells). It is essential to be clear about the reproducibility of results between different experiments, and indeed the results from the 3 or more independent experiments need to be included in the manuscript.
3. My original point 7: siRNA sequences for the different PAR-3 siRNAs are still not included in the methods. Catalog numbers are not adequate because companies change or remove products periodically, and in the future others will not be able to reproduce the data shown here without knowing the sequences of the siRNAs. In addition, the text and figures need to be completely consistent about the naming of the siRNAs (PARD3 or PAR3 or PAR-3 or Par3) and say which siRNA (1 or 2) was used for in Fig. 4. Fig. 4a legend needs to state that cells were transfected with siRNAs, matching it to Fig. EV3 legend. Again, it is essential to state how many cells were analysed from how many different experiments.

4. My original point 10: The revised methods section for PLA is confusing. It would be better to separate the method for exogenously expressed proteins (following transfection) from the method for endogenously expressed proteins.

5. Main changes needed to text/figures point 7: EV3 Figure legend for panel A needs to state that the images are from cells exposed to fluid flow for 60 min.

Referee #3:

Review of revised manuscript from Hikita et al, EMBO reports EMBOR-2017-45253V2, "PAR-3 controls endothelial planar polarity and vascular inflammation under laminar flow"
February 17, 2018

In the revised version of the Hikita et al manuscript EMBOR-2017-45253V2, the authors have made a number of changes to the content and organization of the figures, and to the discussion of their data in the text. However, a number of critical flaws are evident in this version. Please see the following list of comments for a more detailed explanation.

1. The authors evaluate the hypothesis that functional endothelial Par3 equates with intact cell polarity which suppresses inflammation, an important idea highly relevant to the pathology of atherosclerosis. However, in Figure 1 they use vessels in the retina to evaluate endothelial cell (EC) polarity; these ECs are fundamentally different from aortic ECs which are exposed to high shear stress and are not a good tissue for demonstrating the polarity of ECs, as shown by the polarity indexes of wild-type retinal ECs which show low polarity in the Fig. 1C graph. Furthermore, in terms of disease relevance search showing later on retinal vessels particularly different from the proximal aorta in terms of their susceptibility. In the aorta, there is a well-established gradient of polarity depending on the relative position of the cells within the vessel, especially at the aortic arch and branches such as the carotid artery and spine arteries. Their choice of capillary ECs for Fig. 1D is puzzling, since there is very low shear stress in these vessels; and again, ECs are not equivalent in various tissues, especially capillary ECs vs. aortic ECs which are most relevant to atherosclerosis.

2. In Fig. 2 which focuses on inflammation in Par3 EC mutants, there is an overall increase in p65 levels, but it is not limited to the nucleus; rather, in marginal zone and inner curvature p65 is elevated in cytoplasmic and nuclear compartments. How do the authors account for p65 accumulation in the cytoplasm? In active NF kappa B signaling, p65 should shuttle to the nucleus following degradation of its inhibitory subunit and phosphorylation. In Fig. 2D and 2E the VCAM staining is clear and supports increased inflammatory response with loss of endothelial PAR3. However, with the ambiguous p65 result, the overall conclusion regarding inflammation is unclear.

3. Fig.3: The Oil Red O staining in control and Par3 mutant aortas after 10 weeks is very limited given 10 weeks of high-fat diet and ApoE -/- background. Why is the staining so low? It is difficult

to conclude based on these data where levels of athero are so small. In addition, the serum cholesterol levels are lower than expected. The muted increases observed in their athero model raise concerns about these data. Also, the MOMA-2 staining is difficult to see at such low resolution.

4. Fig.4: In panel 4B, why are 293Ts used instead of ECs for westerns? The authors should look at the relevant cell type. Also, why do total GSK3 levels in 4B decrease when there is an increase in phospho-GSK3 in the same samples? And why include the same Rho inhibitor treatment/co-immunoprecipitation of PAR3-PKC for both 293T (4D) and HUVECs (4F?) The co-IP western data for GSK3 with aPKC in 4G is not clean, so it is unclear how the authors quantitated the data.

5. Fig.5: The PLA data is very unclear. There is a lot of overlap between control and inhibitor-treated cells in 4D, and in 4C the low-magnification image for control-18 dyn has cells surrounding the highlighted cell which appear to have the opposite localization of PLA puncta.

6. Finally, there are more relevant in vivo experiments that could be performed such as injection of Rho or GSK inhibitors followed by en face staining or recovery of aortic or lung ECs, that would take advantage of their powerful genetic mouse model to address signaling in the most rigorous and physiological context. The authors did not take full advantage of their best asset in this study.

2nd Revision - authors' response 28 May 2018

Response to reviewers (EMBOR-2017-45253V1)

Referee #1:

The authors have drastically improved the manuscript, which now makes a lot more sense. In general, they have made a good faith effort to unravel a system that is rather complicated. While it isn't perfect, the work is generally done to a high standard. If they can resolve the remaining issues, it would be well worth publishing.

Major criticisms:

The results on aPKC-GSK3b are confusing. They cite literature that,"Previous reports suggest that aPKC, a member of the PAR polarity complex, forms a complex with GSK3ß and controls its activity by phosphorylating Ser9 residue". But their model is that aPKC triggers dephosphorylation of GSK3b, the opposite effect. This needs to be clarified, including some comment about how a kinase mediates dephosphorylation.

Reply

We apologize for making confusion. First of all, we would like to correct our mistake. As the referee pointed out, in previous version of manuscript we mentioned that "Previous reports suggest that aPKC, a member of the PAR polarity complex, forms a complex with GSK3b and controls its activity by phosphorylating S9 residue", however, this was not correct. Previous study showed that GSK3b S9 is upregulated under scratch-induced cell migration (Etienne-Manneville and Hall, Nature, 2003). aPKC forms complex with GSK3b under basal condition, and this complex is dissociated by migratory cue. Because phosphorylated GSK3b was not detected in the aPKC precipitate, the authors mentioned that phosphorylation of GSK3b leads to its dissociation from aPKC. In our system, treating the ECs with 18dyn/cm^2 laminar flow upregulated GSK3b S9 phosphorylation (Fig 4A) and dissociation of the aPKC/GSK3b complex (Figure 4E). Thus, in the last version of revised manuscript, we modified our result section as follows.

P10, lines 228 to 233.

“GSK3b is an ubiquitously-expressed and constitutively-active protein kinase, which was implicated in cytoskeletal reorganization, a number of chronic diseases and inflammation. Phosphorylation of GSK3b at S9 residue downregulates its catalytic activity. Previous report suggests that aPKC, a member of the PAR polarity complex, forms a complex with unphosphorylated active form of GSK3b, and S9 phosphorylation dissociates the complex.”

Fig 5 and EV4. The effect of the Rho kinase inhibitor is analyzed only for the par3-aPKC complex localization to the front. Yet the authors conclude that "that flow-dependent RhoA/Rho kinase activity determines the spatial distribution of PAR-3/aPKCland aPKCl/ GSK3b complexes, which in turn delimits GSK3b activity and microtubule stability". To draw that conclusion, it is necessary to check the

effect of Rho kinase inhibition on polarization of the aPKC/GSK3 complex and ac-tubulin, which they have not done.
According to the referee's comment, we analyzed the effect of Rho-kinase inhibitor on the aPKC/GSK3ß complex formation (Figure 5 E and F) and on the amount of Ac a-Tubulin in the front part of ECs. These data are included in the revised manuscript (Figure 5G and H).

Minor
Intro: it is unclear what they mean by, "Microtubule organization centers (MTOCs) and the Golgi apparatus are aligned parallel to the flow direction". The MTOC and Golgi are usually considered to be in front or behind the nucleus. Parallel and perpendicular don't really apply.
Thank you for pointing out our mistake. To avoid confusion, we modified the sentence as follows (P3, lines 55-57).
"Cell shape is elongated along the axis of flow, and the microtubule organization centers (MTOCs) and the Golgi apparatus are aligned in front or behind the nuclei toward to the flow direction."

Referee #2:

The manuscript is much improved through the authors' additions of new data and new analysis of data, as well as revision of the text and figures to make the methods and analysis clearer.
The remaining points need to be addressed by revisions to the text and figures:

1. My original point 1: add reference(s) and text to the methods section on mice to state that tamoxifen efficiently knocks out PAR3, as previously shown.
Accordingly, we added a reference to the methods section. (P19, line 445).

2. My original point 6: for all graphs showing analysis of cells. The authors still have not stated in most of the figure legends whether the graphs show analysis of cells from just one experiment (in which case statistical analysis is not possible), or whether the analysis is of cells from 3 independent experiments (which have been pooled to give the total number of cells). It is essential to be clear about the reproducibility of results between different experiments, and indeed the results from the 3 or more independent experiments need to be included in the manuscript.
We appreciate the referee for pointing out the problem. In the current version of revised manuscript, we updated quantitation results using mean value of more than three independent experiments (Figures 2C, 5D, 5F, 6B and 6C). We although updated the figure legends and indicated the number of experiments or mice.

3. My original point 7: siRNA sequences for the different PAR-3 siRNAs are still not included in the methods. Catalog numbers are not adequate because companies change or remove products periodically, and in the future others will not be able to reproduce the data shown here without knowing the sequences of the siRNAs. In addition, the text and figures need to be completely consistent about the naming of the siRNAs (PARD3 or PAR3 or PAR-3 or Par3) and say which siRNA (1 or 2) was used for in Fig. 4. Fig. 4a legend needs to state that cells were transfected with siRNAs, matching it to Fig. EV3 legend. Again, it is essential to state how many cells were analysed from how many different experiments.
Thank you for this comment. In the revised manuscript, we added the sequence information for each siRNA (P21 line 502 – 507). In addition, we indicted the siRNA used for each experiment (siPAR-3#1 and #2) in the figures.

4. My original point 10: The revised methods section for PLA is confusing. It would be better to separate the method for exogenously expressed proteins (following transfection) from the method for endogenously expressed proteins.
According to the referee's request, we updated the method section for PLA (P28, line 670 – p29 line 692).

5. Main changes needed to text/figures point 7: EV3 Figure legend for panel A needs to state that the images are from cells exposed to fluid flow for 60 min.
Thank you very much for pointing out our mistake. We include this information in the figure legend (P47 line 1138).

Referee #3:

According to the editor's suggestion, we addressed to the comments #2, 3 and 4 from this referee by text as follows.

2. In Fig. 2 which focuses on inflammation in Par3 EC mutants, there is an overall increase in p65 levels, but it is not limited to the nucleus; rather, in marginal zone and inner curvature p65 is elevated in cytoplasmic and nuclear compartments. How do the authors account for p65 accumulation in the cytoplasm? In active NF kappa B signaling, p65 should shuttle to the nucleus following degradation of its inhibitory subunit and phosphorylation. In Fig. 2D and 2E the VCAM staining is clear and supports increased inflammatory response with loss of endothelial PAR3. However, with the ambiguous p65 result, the overall conclusion regarding inflammation is unclear.

Thank you for pointing out very important issue. As the referee mentioned, the intensity of p65 is increased in the inner curvature and marginal zone of *Pard3*^{iDEC} mice aorta (Figure 2). Previous study showed that disturbed flow increases p65 expression level via JNK1 activation (Cuhlmann et al., Circulation Research, 2011). Interestingly, loss of PAR-3 activates JNK1 in tumor cells (Archibald et al., Oncogene, 2015). These reports suggest that in *Pard3*^{iDEC} mice aorta, nuclear p65 translocation is controlled by PAR-3/GSK3b axis, whereas p65 protein upregulation is mediated by PAR-3/JNK1 pathway. We added this information in the discussion section (P16, line 372-378).

"Additionally, previous study showed that disturbed flow increases the p65 expression level via JNK1 activation and that loss of PAR-3 activates JNK1 in tumor cells[46] . These reports suggest that in *Pard3*^{iDEC} mice aorta, nuclear p65 translocation is controlled by PAR-3/GSK3b axis, whereas p65 protein upregulation may be mediated by the PAR-3/JNK1 pathway."

3. Fig.3: The Oil Red O staining in control and Par3 mutant aortas after 10 weeks is very limited given 10 weeks of high-fat diet and ApoE -/- background. Why is the staining so low? It is difficult to conclude based on these data where levels of athero are so small. In addition, the serum cholesterol levels are lower than expected. The muted increases observed in their athero model raise concerns about these data. Also, the MOMA-2 staining is difficult to see at such low resolution.

In our animal model, it takes 16 weeks to observe obvious atherosclerosis formation. Here, we aimed to show that increased atherosclerosis by loss of PAR-3 in endothelial cells. To address this issue, we focused on the onset of atherosclerosis formation, which was significantly increased in PAR-3 KO mice (Figure 3). In this revised version of manuscript, we mentioned our experimental design as follows (P9 line 197 – 200).

"To analyze the effect of PAR-3 KO on the onset of atherosclerotic plaque formation, mice were sacrificed after 10 weeks of high fat diet feeding. Then aortas were collected and stained with Oil Red O to highlight lipid accumulation."

Thank you for your comment on low resolution of MOMA-2 staining. To improve our manuscript, we replaced panels to higher resolution images (Figure 3E, b and d).

4. Fig.4: In panel 4B, why are 293Ts used instead of ECs for westerns? The authors should look at the relevant cell type. Also, why do total GSK3 levels in 4B decrease when there is an increase in phospho-GSK3 in the same samples?

We have shown that loss of PAR-3 compromised flow-mediated antagonism of PAR-3/aPKC against aPKC/ GSK3b complex formation and thereby increased GSK3b phosphorylation. To complement this loss of function study, we aimed to observe a does-dependent effect of PAR-3 overexpression on GSK3b phosphorylation. Given the row transfection efficiency in cultured endothelial cells, HEK293T cells were adapted for the analysis.

And why include the same Rho inhibitor treatment/co-immunoprecipitation of PAR3-PKC for both 293T (4D) and HUVECs (4F?)

According to the reviewer's suggestion, Figure 4D is moved into Appendix Figure S4.

The co-IP western data for GSK3 with aPKC in 4G is not clean, so it is unclear how the authors quantitated the data.

Sorry for causing confusion. Accordingly, we replaced the panels of Figure 4G with better blots.

2nd Editorial Decision 19 June 2018

Thank you for your patience while we have reviewed your revised manuscript. Your manuscript has been evaluated again by former referee 2 and by myself. As you will see from the report below, this referee is now all 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.

REFEREE REPORT

Referee #2:

The authors have answered all my points comprehensively and no further changes are needed.

2nd Revision - authors' response 20 June 2018

The authors addressed the remaining points.

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: Masanori Nakayama
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45253V1

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?	For in vitro experiments, at least three independent experiments were performed to confirm the reproducibility of the results.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For staining of animal tissues, we used three independent animals for experiment. For atherosclerosis experiments, SD 1,8; Power 80%, Significant difference 0,05 was used as factor and 10 animals were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We used Outlier calculator (https://www.graphpad.com/quickcalcs/Grubbs1.cfm) to exdlude outliers.
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.	All animals were treated with animal caretakers without information of genotype.
For animal studies, include a statement about randomization even if no randomization was used.	All animals were treated with animal caretakers without genotype information.
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.	All animals were treated with animal caretakers without genotype information.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal samples were handled in double-blind manner.
5. For every figure, are statistical tests justified as appropriate?	Yes, we included n number and statistical analysis used in each figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For some experimets with small n number (ex. quantitation of western blotting, n≤5), we employed non-parametric analysis.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	We provided the name of antibodies, company, and product number in method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used HEK 293 ATCC CRL-1573 and Human umbilical vein endothelial cells Pelobiotech PB-200-05f.

* 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.	Pard3 flox/flox; Chd5-CreERT2 B6.129P2-Apoetm1Unc/J59; Pard3 flox/flox; Chd5-CreERT2 Gender: Male Age: P6, P56 or 18 weeks old
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experiments involving animals were conducted in accordance with institutional guidelines and laws, and following the protocols approved by the local animal ethics committees and authorities (Regierungspraesidium Darmstadt, B2/1073 and B2/1122, and Universidade de Lisboa).
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 followed guidelines FELASA (the Federation of Laboratory Animal Science Associations, http://www.felasa.eu/)

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	No.