



# LPHN2 inhibits vascular permeability by differential control of endothelial cell adhesion

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August 13, 2020

Re: JCB manuscript #202006033

Prof. Guido Serini  
Candiolo Cancer Institute, University of Torino  
Strada Provinciale 142 Km 3.95  
Candiolo 10060  
Italy

Dear Dr. Serini,

Thank you for submitting your manuscript entitled "LPHN2 controls vascular morphogenesis by inhibiting endothelial cell adhesion and YAP/TAZ signaling". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. In the reviews from three experts in fields that overlapped the findings reported in this manuscript, there were differences of opinion about the priority of this study for JCB expressed in the comments and recommendations for the Editors. However, our overall conclusion based on the specific comments for authors is that this study could be potentially appropriate for publication in JCB if appropriately revised. Consequently, we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

One of the central questions arising from the reviews is whether or not the in vitro effects on focal adhesions and stress fibers and the in vivo effects on tight junctions are linked or whether LPHN2 acts independent only these structures. The reviewer suggestions below outline possible ways this could be addressed and these experiments would be important. Furthermore, all the reviewers raise some concerns regarding the lack of direct evidence for in vivo morphogenic effects. This will need to be addressed with additional data such as quantifications requested by Rev#2 and better imaging data from the zebrafish KO as requested by Revs#1&3. Depending on these results, please also consider toning down conclusions as requested by Rev#1.

In addition, the suggested experiments on vessel permeability (Rev#3 point 5) and mechanotransduction and YAP (Rev#3 point 2) are important. Rev#1 point 2 suggests an additional haptotaxis assay. This may not be necessary, as the current system is appropriate in our view. However, please provide more details of the system as requested by Rev#3 minor point#1. We agree that the questions from Rev#3 regarding cAMP and RAP1 signaling are interesting but as this is a Report, we feel that experiments to address this would be beyond the scope of this paper.

We strongly encourage you to make every effort to resolve the concerns of these conscientious reviewers.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit.

We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

#### GENERAL GUIDELINES:

Text limits: Character count for a Report is < 20,000; a full Research Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: A Report may include up to 5 main text figures; a full Research Article may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <http://jcb.rupress.org/site/misc/ifora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

\*\*\*IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.\*\*\*

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures; a full Research Article may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Johanna Ivaska, Ph.D.  
Monitoring Editor  
Journal of Cell Biology

Dan Simon, Ph.D.  
Scientific Editor  
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

Camillo et al. investigated the role of LPHN2, an adhesion G protein-coupled receptor, in vascular morphogenesis. They proposed that LPHN2 localizes at ECM adhesion in endothelial cells (ECs) and signals through cAMP/Rap1 to negatively regulate haptotaxis. Moreover, the authors suggested that this pathway negatively controls YAP/TAZ1 activity in ECs. They further proposed that this mechanism is dependent on the cell-autonomous expression of FLRT2, a key LPHN2 ligand. In addition to the in vitro data, Camillo et al. generated a *lphn2a* mutant zebrafish line. They proposed that ECs are abnormally stretched, display enhanced YAP/TAZ activation and that ECs have defective intercellular junctions. They also showed that zebrafish *lphn2a* null embryos facilitate cancer cell extravasation compared to control animals. Finally, they concluded that they uncovered a role for LPHN2 in vascular development that could be exploited to interfere with cancer metastatic dissemination.

Overall, this study provides an interesting new crosstalk between LPHN2 and Hippo pathways, which seems important to regulate ECs migration. However, several conclusions are only weakly supported by the data. Authors tended to overinterpret and to over conclude from their experiments, and the link between in vivo (permeability phenotype) and in vitro (migration phenotype) data is very difficult to interpret. Therefore, the reviewer does not support the publication of the current version of this MS. Nevertheless, the reviewer provide some specific comments that might be helpful for the authors.

1. The authors tend to over conclude from their experiments, and therefore they should be more careful when discussing their results. For instance, authors wrote as summary of their work "To conclude, we identified the endothelial ADGR LPHN2 as a novel repulsive guidance receptor that controls in vivo vascular morphogenesis and function." Authors do not show any repulsive guidance effects neither any in vivo vascular morphogenic defects.

As another example, in p.5, they state "Thus, in cultured ECs LPHN2 mediates inhibitory signals that are likely initiated by autocrine loops of endogenous LPHN2 ligands". None of the experiments shown can allow to deduce if the effect on haptotaxis is indeed autocrine, it could be due to paracrine signals or junction mediated signals. Or p.7 they conclude "Hence, in ECs LPHN2 inhibits FA and stress fiber formation along with the ensuing nuclear translocation of YAP/TAZ that controls gene transcription in response to mechanosignaling". Although some links have been demonstrated by the literature between YAP/TAZ pathway and mechanosignaling, here the authors only demonstrate that LPHN2 knock down induces FA and stress fiber formation and YAP/TAZ translocation to the nucleus. They do not demonstrate any causality in this specific context, only a correlation between different phenomena.

2. The conclusions on the role of LPHN2 in cell adhesion and haptotaxis are exclusively done using a single type of assay, a commercial impedance-based haptotaxis assay. Authors should reproduce their results in a different type of assay, more representative of haptotaxis. If LPHN2 controls the ability of adhere to the upper chamber or to pass through the porous membrane, one would expect to have a similar output in terms of the number of cells located in the bottom chamber, whilst the mechanism would be substantially different.

3. Further analyses on the effect of LPHN2 knock down on FA and stress fibers are needed (localization, structure, size) to bring a better understanding of the phenotype.

4. The zebrafish images are difficult to analyze, higher magnifications are needed to convince the

reader of the quantification made. LPHN2 enrichment in the PCV is not obvious in the images either. Moreover, the authors state the animals display an "aberrant vascular phenotype" after demonstrating that vascular patterning and blood flow are normal which is highly incoherent. Moreover, are zebrafish mutants viable? For these experiments, do authors cross HET x HET to get WT and KO from the same batch, or WT and KO animals are from different parents?

5. It is very much unclear the link between haptotaxis and cancer extravasation. Those two phenomena are not linked in the MS. It is also unclear the role of YAP/TAZ activation in any of those phenotypes.

Some minor issues.

1. Introduction should include background on LPHNs, and on LPHN2 in particular.
2. All graphs in the MS should display all data points with SD, not SEM.
3. Immunofluorescence images provided are often overexposed. Some should display a higher magnification image like Figure S3, for which it is difficult to visualize properly YAP and TAZ nuclear translocation.
4. Why authors used soft hydrogels (5kPa) to assess YAP activity is not clear.
5. Authors should provide additional stainings for FAs, not only vinculin.
6. Figure 2D, controls are missing (siCtrl with both WT and  $\Delta$ LOF) and should be displayed at least in supplementary data.
7. Does addition of exogenous FLRT2 elevate Rap1 levels in control cells?
8. Does siFLRT2 change LPHN2 localization?
9. Figure 5B, colors make the image difficult to analyze.
10. Figure 5C does not exist.
11. In Figure 4C, the tight junction analysis is over interpreted, and images are not convincing as not enough junctions are shown. No quantification is shown to support the description.
12. In the extravasation study, images don't show clearly what is intra and extra vascular, making the reader not easily convinced by the authors' statements.

Reviewer #2 (Comments to the Authors (Required)):

This is an interesting and important paper on the coordinated regulation of mechanical sensing and morphogenesis in vascular endothelial cells. The authors have uncovered an entirely novel pathway in endothelial signalling whereby LPHN2 acts at focal adhesions to suppress endothelial motility and mechanical sensing through the YAP/TAZ pathway, while promoting tight junction formation. The authors also show that endothelial cells express the LPHN2 ligand FLRT2, and that this creates a functional autocrine loop.

The experimental work is of a high standard. Either the work has been through previous review, or the authors have anticipated reviewer requests - there is very little improvement to suggest.

I had some concerns about the use of a single siRNA for LPHN2, but the key findings are supported by rescues, and the work is validated in a zebrafish model through a different genetic approach.

I think that the morphological changes seen in vivo are important. If it was possible to quantify some of the changes shown in Figures 4B and 4C, that would be great. If this is not possible, maybe the authors could reword this section to be clear that the observed changes require quantification for certainty. I think this would be enough, as there is quantitative work on the in vivo YAP/TAZ outcomes and the extravasation.

I wondered if FLRT2 could act in cis with LPHN2 on endothelial cells, or if it needed to be shed. And if it could act in cis, I wondered if there might be separation of the ligand and receptor in polarised EC monolayers - a bit like the separation of EGFR and its ligand in epithelial cells in airways. That would be interesting in cases where the barrier is disrupted and polarity is temporarily lost.

And it also seemed that there was potential for communication between neurons and endothelial cells during development, with the neurons presenting an FLRT ligand. That might be observable in movies of the zebrafish - as changes to the elongation of endothelial cells along neurons and/or rates of migration along them.

I don't need either of those points addressed by experimental work for revision. I think it is a mark of the value of the study that it immediately suggests further work.

The position of endothelial cells as an interface in the body means that they must constantly survey their environment and interact with other cell types to effect appropriate changes to morphology and tension. This paper makes a significant contribution to our understanding of these fundamental cell processes.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Camillo and colleagues describe the function of the LPHN2 adhesion G-protein-coupled receptor in human and fish endothelial cells. They provide evidence that LPHN2 is located at focal adhesions, where it promotes FA stability. In so doing it also slows down EC migration, and facilitates activation of YAP, used here as a read-out of adhesion "strength". LPHN2 is activated by FLRT2, expressed by EC cells themselves, indicating an autocrine signalling. In zebrafish embryos, inactivation of LPHN2a induced some endothelial phenotypes, including incomplete TJ formation and increased leakiness of vessels to metastatic cells.

Major comments:

1) The authors provide data on the function of LPHN2 in cultured ECs based on the use of a single siRNA. The use of a single siRNA can have problems associated to off-target and non-specific effects. The authors do provide the rescue with haptotaxis, but it would be important to provide a similar rescue with other key functional data (vinculin and stress fibers, YAP assays etc.). Similarly, it would be nice to see recombinant FLRT2 rescuing siFLRT2 in migration assays.

2) Given the FA- and stress-fiber-inhibitory role of LPHN2, the authors explore whether LPHN2 regulates YAP mechanotransduction. For this, they use hydrogels of intermediate stiffness (5kPa), and show increased nuclear intensity in YAP and TAZ IF, accompanied by increased CTGF expression. Controls are lacking to gauge the effect of 5kPa on YAP - usually, a soft ECM induces the translocation of YAP to the cytoplasm, which is not observed here. This is perhaps related to the general observation that 5kPa is an intermediate situation inducing minor YAP inactivation (see Eloegui-Artola NCB 2016 and Totaro NCOMMS 2017), but it raises some doubts that the soft condition used here is relevant.

Please also note on this point that not all GPCR have the same effects: some activate YAP through RHO, some inhibit YAP through PKA (see Guan paper in G&D). Thus, regulation of cAMP might be well in line with an inhibitory GPCR and explain YAP inhibition, independently of FAs and stress-

fibers. If so, the relevance of using hydrogels and YAP mechanoresponses becomes less interesting. In addition, it would also be valuable to understand whether LPHN2 also plays a role in the context of a confluent EC monolayer, or only in the context of isolated motile EC cells, also in light of the fish phenotypes where ECs are fully "confluent".

3) The description of the phenotype in LPHN2aKO embryos does not match the images. Vessels (which vessels? all? in the picture, multiple specific vessels are shown, and is unclear to which vessel the lowest panel refers to, and in which vessel(s) the phenotype is observed) should be thinner and longer, but this is not so apparent from the pictures and no quantification of this phenotype is provided. The same applies to TEM images, where the difference in EC thickness is not so apparent.

4) Data in embryos suggest a role for LPHN2a in vessel formation and in regulation of Hippo. However, whether the two are causally related (as suggested by the authors) or just two associated events (for example, loss of cell-cell adhesions might be driving Hippo signalling) remains unknown. Please revise the claims and discussion accordingly.

5) Data on enhanced vessel permeability are nice, but need to be complemented by a more standard dextran injection. If TJ are directly affected (as opposed to the ability of ECs to interact with metastatic cells, for example), dextran should be leaky.

6) It remains unclear how the proposed function of LPHN2 in regulating EC-to-ECM adhesions (shown in vitro) relates to the in vivo TJ phenotype. Is this also observed in vitro, with confluent monolayers (e.g. transendothelial conductance assays, or fluorescent dextran permeability)? Is this dependent on cAMP and RAP1 signaling? and/or on Hippo?

Minor points:

Please describe better the haptotaxis assay - it is not clear why the authors state "migration towards collagen", when in my understanding what is being measured is the displacement on collagen-coated dishes. Accordingly, provide a better definition of the y-axis in the migration assays (what does relative mean? relative to the initial position? what is the measure, microns? is the measure the total path, or the radius from the initial position?). In the text, the same assays are also described as "directional migration" (figure 3) but the assay is the same, and it is not clear why this assay is particularly instructive on the directionality. Is this measuring migration persistence? Please clarify and use terms accordingly. This also related to the use of "chemorepulsion" - it is unclear whether the phenotypes are just related to migration speed (as the reviewer understands), or to migration directionality (which would imply some kind of asymmetric source of FLRTs?).

When introducing FLRT as possible ligands of LPHN2, this comes unexpected because in the introduction LPHN2 are described as ECM receptors. Please describe better in the introduction.

Please describe better in the text the "ligand-receptor in situ binding assay" by indicating the use of recombinant FLRT2

When describing LPHN2 expression in zebrafish, it is said it is enriched in vascular ECs of the DA and PCV, but compared to what? to the surrounding tissues, or to other EC? In other words, is LPHN2 expressed mainly in these vessels and not in others, or in all ECs). Technically, is the antibody epitope conserved? Is the staining lost in LPHN2aKO?

Please briefly introduce what the Tg(kdrl:EGFP)s843 line is used for, for the sake of non-experts

Is the choice of LPHN2a mutagenesis dictated by chance (vs. possible LPHN2b, c, d etc)? In other words, is there room for functional redundancy with other duplicated genes, so that one may expect stronger phenotypes by complete inactivation of LPHN2s? This should be discussed.

Why ECs are not described with their more common name HUVECs?

Please provide the complete siRNA sequences in the methods including controls if possible.



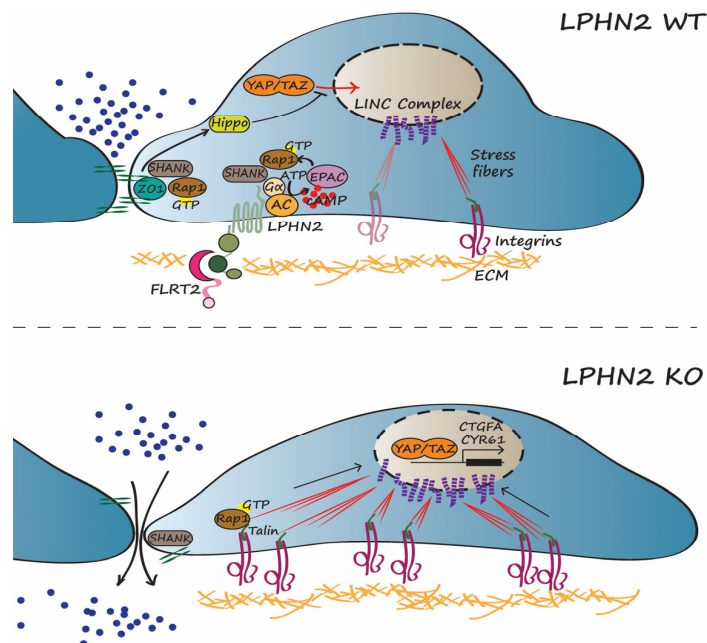
Editors

1. One of the central questions arising from the reviews is whether or not the *in vitro* effects on focal adhesions and stress fibers and the *in vivo* effects on tight junctions are linked or whether LPHN2 acts independent only these structures. The reviewer suggestions below outline possible ways this could be addressed and these experiments would be important.

We provide new data showing that in cultured endothelial cells (ECs) LPHN2 signaling, in addition to activating Rap1 GTP loading and negatively regulating focal adhesion (FA) and stress fiber formation, promotes the targeting of the key adaptor ZO-1 to tight junction (TJs) (Fig. 5A-B).

Thanks to our new data on LPHN2 impact on TJs and recently published studies on the role of Rap1 in TJ assembly (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088), we can now more robustly discuss and provide a clearer link between LPHN2 signaling, cell-to-ECM adhesions and TJs. In particular, the small GTPase Rap1, which is a well-known regulator of cell-to-ECM adhesions (Larrigue et al., 2016, *Blood*, 128:479-487; Colò et al., 2012, *J. Cell Sci.* 125: 5338-5352) and to increase EC barrier function (Bos, 2018, *Cold Spring Harb. Perspect. Med.*, 8:a031468), very recently was formally showed to promote the formation of TJs (Sasaki et al., 2020, *Cell Rep.* 31:107407), which in ECs play a crucial role in the control of vascular permeability. Therefore, it is conceivable that LPHN2 activation of Rap1 acts both to inhibit the formation of FAs and to promote the assembly of TJs, which increase EC barrier function.

Further work is required to thoroughly understand the mechanisms by which LPHN2 exerts, via Rap1, its opposite effect on FAs and TJs. However, as discussed in the last part of our revised manuscript (and depicted in the schematic below), the ability of LPHN2 to directly bind the central PSD-95/Dlg/ZO-1 (PDZ) domain of the SH3 and multiple ankyrin repeat domains (SHANK) proteins is in this regard likely crucial. Indeed, the binding of the N-terminal Shank/ProSAP N-terminal (SPN) domain of SHANK to Rap1-GTP was found both to: i) suppress talin-mediated integrin activation and FA development (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305); ii) promote the assembly of TJs (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088). Therefore, LPHN2 may favor the turnover of FAs and the assembly of TJs by funneling Rap1-GTP towards SHANK.



2. Furthermore, all the reviewers raise some concerns regarding the lack of direct evidence for *in vivo* morphogenic effects. This will need to be addressed with additional data such as quantifications requested by Rev#2 and better imaging data from the zebrafish KO as requested by Revs#1&3. Depending on these results, please also consider toning down conclusions as requested by Rev#1.

We further characterized the morphological features of the *in vivo* vascular phenotype of *lphn2a* knockout zebrafish embryos. Indeed, together with the previously reported lack of intercellular junctions among ECs and increased cancer cell extravasation, we quantified and showed that, compared to wild type animals, *lphn2a* knockout zebrafish embryos display: i) significantly decreased EC cell surface area, as evaluated by confocal microscopy Z-sectioning and 3D reconstruction of trunk blood vessels (Fig. 4E); ii) evident decreased EC area/blood vessel perimeter ratio, as evaluated in electron microscopy (Fig. 4F).

We also worked to eliminate from the text any potential statement that might be perceived as an over interpretation of showed experiments and to discuss our results more carefully.

3. In addition, the suggested experiments on vessel permeability (Rev#3 point 5) and mechanotransduction and YAP (Rev#3 point 2) are important.

We quantified and showed that, compared to wild type animals, *lphn2a* knockout zebrafish embryos display a robust increased extravasation of intravascularly injected 70 kDa FITC-dextran both in basal condition and also upon stimulation of vascular permeability by treatment with exogenous VEGF (Fig. 5C).

Based on previous reports (Birukova et al., 2013, *Microv. Res.*, 87:50–57; Galie et al., 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, Stiffness sensing by cells, 2020, *Physiol. Rev.*, 100:695-724), we repeated the experiments and identified 10 kPa as an optimal stiffness for cultured EC monolayers. Following the suggestions of Reviewers and Editors, and taking into account previous findings by Pere Roca-Cusachs and collaborators (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548) that ECM coating density is a key determinant of force transmission at integrin-based adhesion sites, we performed a series of new quantifications to evaluate YAP and TAZ nuclear translocation in both control and LPHN2 silenced ECs that were also: i) transduced or not with exogenous wild type or mutant  $\Delta$ OLF LPHN2 constructs; ii) plated on 10 kPa rigid substrates pre-coated with increasing amounts (1, 3, and 5  $\mu$ g/ml) FN. In agreement with Elosegui-Artola et al. (*Nat. Cell Biol.*, 2016, 18:540-548) we found that on 10 kPa rigid substrates, increasing amounts (1, 3, and 5  $\mu$ g/ml) of FN resulted in a dose-response translocation of the key mechanosensory effectors YAP/TAZ in the nucleus of control ECs. Furthermore, we found that, the increased number and size of cell-to-ECM adhesions and actin stress fibers correlated with a higher nuclear translocation of YAP and TAZ in LPHN2 silenced ECs, compared to control silenced ECs (Fig. 1K-L). Of note, such an increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was rescued by the introduction of exogenous wild type LPHN2, but not by the  $\Delta$ OLF LPHN2 mutant construct which is incapable of binding FLRT2 (Fig. 2G-H).

4. Rev#1 point 2 suggests an additional haptotaxis assay. This may not be necessary, as the current system is appropriate in our view. However, please provide more details of the system as requested by Rev#3 minor point#1.

On page. 18-19, we provide additional information on the employed impedance-based haptotactic migration system originally produced by ACEA Biosciences/Agilent Technologies.

*5. We agree that the questions from Rev#3 regarding cAMP and RAP1 signaling are interesting but as this is a Report, we feel that experiments to address this would be beyond the scope of this paper.*

We agree with the Reviewer#3 and the Editors. We were aware of the signaling pathway originally reported by the Guan lab (Yu et al., 2013, *Genes & Dev.* 27:1223-1232), which was reported as reference 106 in the review manuscript by Piccolo and collaborators (Totaro et al., 2018, *Nature Cell Biol.* 20: 888-899) and also cited in our manuscript. In their 2013 paper, Yu and colleagues show how GPCRs can: i) activate YAP/TAZ via G $\alpha$ 12/13, q/11-dependent activation of RHO; ii) inhibit YAP/TAZ via G $\alpha$ s/AC/cAMP/PKA-dependent inhibition of RHO. For this reason, as shown in [Fig. S11](#) and discussed on [page 6 \(first paragraph\)](#), we carefully investigated this aspect and we did not detect any reduction in RHO activation after LPHN2 silencing in ECs.

## Referee #1:

### Major points:

1A. The authors tend to over conclude from their experiments, and therefore they should be more careful when discussing their results. For instance, authors wrote as summary of their work "To conclude, we identified the endothelial ADGR LPHN2 as a novel repulsive guidance receptor that controls in vivo vascular morphogenesis and function." Authors do not show any repulsive guidance effects neither any in vivo vascular morphogenic defects.

We worked to **eliminate** from the text any potential statement that might be perceived as an **over interpretation** of showed experiments and to **discuss** our results **more carefully**.

Concerning the specific statement "To conclude, we identified the endothelial ADGR LPHN2 as a novel repulsive guidance receptor that controls in vivo vascular morphogenesis and function", we agree with the reviewer (see also minor point 11) that **the lack of latrophilin 2 (LPHN2) in vivo results in defects of blood vessel and EC function** that differ from those often caused by the knockdown of "classical" repulsive guidance cues. We hypothesize, and in part demonstrated in this work revision, that such phenotypic differences are likely due to the **unique and multifaceted signaling cascade events and biological effects** that we found elicited by LPHN2 *in vitro*. Indeed, we found that FLRT2-activated LPHN2: i) inhibits the formation of cell-to-extracellular matrix (ECM) adhesions and actin stress fibers; ii) inhibits stiffness- and ECM-elicited YAP/TAZ nuclear translocation and signaling; iii) promotes the stiffness- and ECM-elicited localization of the key tight junction adaptor ZO-1 at cell-to-cell contacts. **Moreover**, we showed that, analogously to receptors that transduce signals of other guidance cues in endothelial cells (ECs) [e.g. Plexin A4 receptor and semaphorin 3A (SEMA3A), Gioelli et al., 2018, *Science Transl. Med.*, 10: eaah4807], **LPHN2 mediates the inhibitory (chemorepulsive) effects of its well-known ligand FLRT2** (reviewed in Seiradake et al., 2016, *Annu. Rev. Cell Dev. Biol.* 32: 28.21–28.32) on the haptotactic migration of ECs towards a solid phase digital gradient of ECM. We therefore conclude that, similarly to FLRT3 ligand-dependent activation of LPHN3 in neurons (Seiradake et al., 2016, *Annu. Rev. Cell Dev. Biol.*), LPHN2 acts as a receptor transducing the repulsive guidance effects of the FLRT2 ligand in ECs.

1B. As another example, in p.5, they state "Thus, in cultured ECs LPHN2 mediates inhibitory signals that are likely initiated by autocrine loops of endogenous LPHN2 ligands". None of the experiments shown can allow to deduce if the effect on haptotaxis is indeed autocrine, it could be due to paracrine signals or junction mediated signals.

The sentence which the Reviewer #1 is referring to is hypothetical. It was indeed formulated to introduce the reader to the reason why we decided to look for the presence of LPHN2 ligand(s) produced by ECs themselves. We however agree with the reviewer that the LPHN2-ligand FLRT2 may act in both an autocrine/paracrine manner in cultured ECs, so **we modified the text accordingly (see page 8, first paragraph)**.

We wondered whether, similarly to Reviewer #2, Reviewer #1 had also thought that FLRT2 may act in cis with LPHN2 on ECs, or if it may need to be shed. Based on our finding that, in control cells, LPHN2, but not FLRT2, is highly enriched at ECM adhesion sites, we hypothesized that FLRT2 ligand must be shed to activate at least the large fraction of LPHN2 that concentrates in EC-to-ECM adhesion contacts. However, we also considered that the uncleaved FLRT2 may activate LPHN2 localized outside ECM adhesions. **We discussed this possibility on page 8, first paragraph**.

1C. Or p.7 they conclude "Hence, in ECs LPHN2 inhibits FA and stress fiber formation along with the ensuing nuclear translocation of YAP/TAZ that controls gene transcription in response to mechanosignaling". Although some links have been demonstrated by the literature between YAP/TAZ pathway and mechanosignaling, here the authors only demonstrate that LPHN2 knock down induces FA and stress fiber formation and YAP/TAZ translocation to the nucleus. They do not demonstrate any causality in this specific context, only a correlation between different phenomena.

We showed how: i) **LPHN2 inhibits the formation of cell-to-ECM adhesions and actin stress fibers**; ii) in LPHN2 silenced ECs, the increased number and size of cell-to-ECM adhesions and actin stress fibers correlated with a **higher nuclear translocation of the key mechanosensory effectors YAP and TAZ** (Fig. 1K, L). Of note, such increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was **rescued by the introduction of exogenous wild type LPHN2, but not by the  $\Delta$ OLF LPHN2 mutant** construct which is incapable of binding FLRT2 (Fig. 2G, H).

We interpreted our data based on the **literature showing and describing YAP/TAZ as key mechanosensory effectors** in mammalian cells, ECs included, as summarized in recent reviews top ranking journals, such as:

- Kechagia, Ivaska & Roca-Cusachs. Integrins as biomechanical sensors of the microenvironment. 2019, *Nature Rev. Mol. Cell Biol.*, 20: 457–473.
- Moya & Halder. Hippo–YAP/TAZ signalling in organ regeneration and regenerative medicine. 2019, *Nature Rev. Mol. Cell Biol.*, 20: 211–226.
- Panciera, Azzolin, Cordenonsi & Piccolo. Mechanobiology of YAP and TAZ in physiology and disease. 2018, *Nature Rev. Mol. Cell Biol.*, 20: 211–226.

2. *The conclusions on the role of LPHN2 in cell adhesion and haptotaxis are exclusively done using a single type of assay, a commercial impedance-based haptotaxis assay. Authors should reproduce their results in a different type of assay, more representative of haptotaxis. If LPHN2 controls the ability of adhere to the upper chamber or to pass through the porous membrane, one would expect to have a similar output in terms of the number of cells located in the bottom chamber, whilst the mechanism would be substantially different.*

Haptotaxis assays rely on continuous or digital gradients and home-made or commercially available experimental systems. We (Serini et al., *Nature*, 2003, 424:391-397; Gioelli et al., 2018, *Science Transl. Med.*, 10:eaah4807) and others (e.g. Yang et al., 2017, *EMBO Mol. Med.*, 7:1267-1284) have always been studying and describing the impact of repulsive guidance cues on EC haptotaxis by employing digital gradients generated by coating the lower side of an 8  $\mu$ m pore size filters (ideal for migrating ECs) with ECM proteins. The recent employment of a state-of-the art impedance-based system allowed us to precisely and reproducibly monitor the impact of Semaphorin 3A signaling *via* Neuropilin 1 and Plexin A4 receptor on haptotactic EC migration towards the ECM (Gioelli et al., 2018, *Science Transl. Med.*, 10:eaah4807).

In this regard, **JCB Monitoring and Scientific Editors commented**: "Rev#1 point 2 suggests an additional haptotaxis assay. This may not be necessary, as the current system is appropriate in our view. However, please provide more details of the system as requested by Rev#3 minor point#1." Therefore, **on page. 18-19, we provide additional information on the employed impedance-based haptotactic migration system** originally produced by ACEA Biosciences/Agilent Technologies.

3. Further analyses on the effect of LPHN2 knock down on FA and stress fibers are needed (localization, structure, size) to bring a better understanding of the phenotype.

(See also major point 5).

As requested, we provide further characterization of the impact of **LPHN2 knock-down and rescue with silencing resistant LPHN2 wild type (WT)** and  $\Delta$  olfactomedin domain ( **$\Delta$ OLF, incapable of binding FLRT2**) mutant constructs on focal adhesion (FA) and stress fibers (see [page 7](#), [Fig. 2A-F](#) and [Fig. S2D-F](#)). We stained FAs for both vinculin and paxillin and then imaged by confocal microscopy and quantified their number (normalized on cell area) and maximum Feret's diameter. We found that **in ECs LPHN2 silencing** results in a statistically significant **2-fold increase** of **vinculin<sup>+</sup>** ([Fig. 1F-H](#)) and **paxillin<sup>+</sup>** ([Fig. S1F](#)) **FA number** and maximum **Feret's diameter**, which were **rescued by** silencing resistant **LPHN2 WT**, but **not LPHN2  $\Delta$ OLF** ([Fig. 2A-C](#) and [Fig. S2D-F](#)). In addition to the number of phalloidin stained stress fibers normalized on the cell area, we employed high resolution 3D stimulated emission depletion (STED) confocal microscopy along the XZ plan to carefully measure the mean cross-sectional area of stress fibers. We found that **LPHN2 silencing** results in a statistically significant **increase** in the **number (2.5-fold)** and XZ **cross-sectional area (2-fold)** of stress fibers, which were **rescued by** silencing resistant **LPHN2 WT**, but **not LPHN2  $\Delta$ OLF** ([Fig. 2A-F](#) and [Fig. S2D-F](#)).

4A. The zebrafish images are difficult to analyze, higher magnifications are needed to convince the reader of the quantification made. LPHN2 enrichment in the PCV is not obvious in the images either.

We performed a **new** series of stainings with **anti-LPHN2** antibodies followed by **confocal fluorescence microscopy** analyses on transversal sections of **wild type** and ***lphn2a* knock-out** zebrafish embryos with ***Tg(kdrl:EGFP)<sup>S843</sup>*** genetic background in which EGFP is selectively expressed in ECs. In [Fig. 4A](#), we show that **LPHN2** is expressed in EGFP<sup>+</sup> ECs of both dorsal aorta (**DA**) and pericardinal vein (**PCV**) of wild type (arrows), but not ***lphn2a* knock-out** zebrafish embryos. Furthermore, **high magnification areas** are shown.

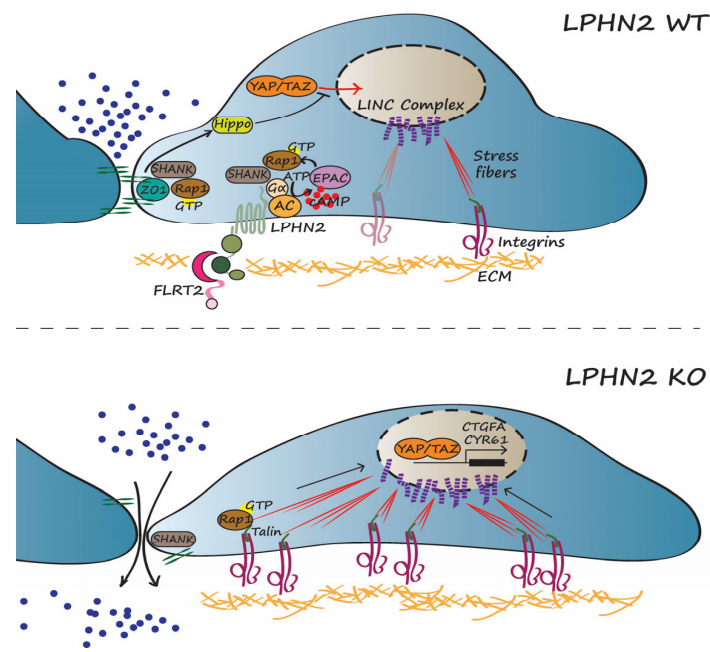
4B. Moreover, the authors state the animals display an "aberrant vascular phenotype" after demonstrating that vascular patterning and blood flow are normal which is highly incoherent. Moreover, are zebrafish mutants viable? For these experiments, do authors cross HET x HET to get WT and KO from the same batch, or WT and KO animals are from different parents?

While we did not observe major vascular anomalies in terms of blood vessel patterning and blood flow, we did find **aberrations** at the level of **EC intercellular adhesions**, cell **shape** (height and length) and **function** (cancer cell extravasation). These are the reasons why we previously defined the vascular phenotype as aberrant. In addition, we **further characterized** these aspects in our revised manuscript. Indeed, **we quantified and showed** that, compared to wild type animals, ***lphn2a* knockout zebrafish embryos** display: i) significantly decreased **EC cell surface area**, as evaluated by confocal microscopy Z-sectioning and 3D reconstruction of trunk blood vessels ([Fig. 4E](#)); ii) evident decrease in the **EC area/blood vessel perimeter ratio**, as evaluated in electron microscopy ([Fig. 4F](#)); iii) a robust increased **extravasation** of intravascularly injected **70 kDa FITC-dextran** both in **basal** condition and also upon stimulation of vascular permeability by treatment with exogenous **VEGF** ([Fig. 5C](#)). In all the experiments we used homozygous larvae obtained from heterozygous parents coming from different batch of eggs.

5. It is very much unclear the link between haptotaxis and cancer extravasation. Those two phenomena are not linked in the MS. It is also unclear the role of YAP/TAZ activation in any of those phenotypes.

We showed that **LPHN2 promotes the GTP-loading and activation of the small GTPase Rap1**, which is a well-known regulator of **cell-to-ECM adhesions** (Larrigue et al., 2016, *Blood*, 128:479-487; Colò et al., 2012, *J. Cell Sci.* 125: 5338-5352). While known for its ability to signal and increase **EC barrier function** (Bos, 2018, *Cold*

*Spring Harb. Perspect. Med.*, 8:a031468), **Rap1** was only very recently formally showed to **promote the formation of tight junctions** (TJs; Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088), which in ECs play a crucial role in the control of vascular permeability. Therefore, as discussed on [pages 10-11](#), it is **conceivable that LPHN2 activation of Rap1 acts both to inhibit the formation of focal adhesions (FAs) and to promote the assembly of TJs**, which increase EC barrier function. Further work is required to thoroughly understand the mechanisms by which LPHN2 exerts, *via* Rap1, its opposite effect on FAs and TJs. However, as discussed in the last part of our revised manuscript (and depicted in the *schematic* below, corresponding to [Fig. 5E](#)), **the ability of LPHN2 to directly bind the central PSD-95/Dlg/ZO-1 (PDZ) domain of the SH3 and multiple ankyrin repeat domains (SHANK) proteins is in this regard likely crucial**. Indeed, the binding of the N-terminal Shank/ProSAP N-terminal (SPN) domain of **SHANK to Rap1-GTP** was found both to: i) **suppress talin-mediated integrin activation and FA development** (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305); ii) **promote the assembly of TJs** (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088). Therefore, LPHN2 may favor the turnover of FAs and the assembly of TJs by funneling Rap1-GTP towards SHANK.



Concerning YAP/TAZ, while **TJs inhibit** the nuclear translocation of **YAP and TAZ** through their Hippo pathway-dependent phosphorylation, **FAs** and the associated F-actin **stress fibers** exert exactly the opposite effect **promoting YAP/TAZ** nuclear localization and transcriptional function (Karaman and Halder, 2018, *Cold Spring Harb. Perspect. Biol.*, 10:a028753; Moya & Halder, 2019, *Nature Rev. Mol. Cell Biol.*, 20: 211–226). As discussed on [page 11](#), we then posit that the nuclear translocation and functional **activation of YAP/TAZ**, observed upon LPHN2 silencing/knockdown *in vitro* ([Fig. 1K-M](#)) and *in vivo* ([Fig. 4C-D](#)), lie **downstream of the increased formation of FAs/stress fibers** and the disassembly of **TJs**. Of note, such an increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was **rescued by the introduction of exogenous wild type LPHN2, but not by the ΔOLF LPHN2 mutant** construct which is incapable of binding FLRT2 ([Fig. 2G-H](#)).

An additional aspect concerning the cross-talk between FAs and TJs emerged quite recently from studies of the laboratories of Karl Matter and Maria Balda revealing how **ECM physical properties drive** the targeting of the cytoskeletal adaptor **ZO-1 at TJs, thus influencing their assembly** (Haas et al., *Cell Rep.*, 2020, 32: 107924). Therefore, based on previous reports (Birukova et al., *Endothelial barrier disruption and recovery is controlled by substrate stiffness, Microv. Res.*, 2013, 87:50–57; Galie et al., *Application of multiple levels of fluid shear stress to endothelial cells plated on polyacrylamide gels*, 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, *Stiffness sensing by cells*, 2020, *Physiol. Rev.*, 100:695-724), we first

identified **10 kPa as an optimal stiffness for cultured EC monolayers**. Next, we plated ECs on 10 kPa rigid substrates pre-coated with **increasing amounts (1, 3, and 5 µg/ml) of fibronectin (FN)**. Indeed, Pere Roca-Cusachs and collaborators (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548) first showed that **ECM coating density** is a **key determinant of force transmission at integrin-based adhesion sites**. In agreement with the mentioned studies by Matter, Balda and Rocha-Cusachs, we observed how increasing amounts of FN result in a **progressive dose-dependent targeting of ZO-1 at endothelial cell-to-cell contacts in control, but not in LPHN2 silenced cells (Fig. 5A)**. The lack of FN-coating density-dependent ZO-1 translocation caused by the silencing of endogenous LPHN2 was **rescued by** the introduction of exogenous **wild type LPHN2, but not** by the **ΔOLF LPHN2 mutant** construct which is incapable of binding FLRT2 (**Fig. 5B**).

*Minor points:*

1. *Introduction should include background on LPHNs, and on LPHN2 in particular.*

Compatibly with text limits of *JCB* Report manuscript format, we provided additional background on LPHN2 in the *Introduction* section (**page 4**).

2. *All graphs in the MS should display all data points with SD, not SEM.*

**We modified all the *in vitro* graphs** as all data dot plots with **means and SD**.

3. *Immunofluorescences image provided are often overexposed. Some should display a higher magnification image like Figure S3, for which it is difficult to visualize properly YAP and TAZ nuclear translocation.*

We performed a series of new experiments in which we visualized YAP and TAZ nuclear translocation at both **low and high magnification** in both control and LPHN2 silenced cells in which exogenous **wild type or mutant ΔOLF LPHN2** constructs were also introduced to evaluate the ability to **rescue the higher YAP/TAZ nuclear translocation** phenotype caused by LPHN2 silencing. These data are reported in new **Figs. 1K-L** and **Fig. 2G-H**, showing that the increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 (**Figs. 1K-L**) is **rescued by** the introduction of exogenous **wild type LPHN2, but not** by the **ΔOLF LPHN2 mutant** construct which is incapable of binding FLRT2 (**Fig. 2G, H**).

4. *Why authors used soft hydrogels (5kPa) to assess YAP activity is not clear.*

Based on previous reports (Birukova et al., 2013, *Microv. Res.*, 87:50–57; Galie et al., 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, Stiffness sensing by cells, 2020, *Physiol. Rev.*, 100:695-724), we repeated the experiments and identified **10 kPa as an optimal stiffness for cultured EC monolayers** and performed a series of new quantifications to evaluate YAP and TAZ nuclear translocation in both control and LPHN2 silenced cells that were also transduced or not with exogenous **wild type or mutant ΔOLF LPHN2** constructs. Next, since **ECM coating density** was previously identified as a **key determinant of force transmission at integrin-based adhesion sites** (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548), we plated ECs on 10 kPa rigid substrates pre-coated with **increasing amounts (1, 3, and 5 µg/ml) FN**.

5. *Authors should provide additional stainings for FAs, not only vinculin.*

As reported in our reply to main point # 3, we stained FAs for both **vinculin** and **paxillin** and then imaged by confocal microscopy and quantified their number (normalized on cell area) and maximum Feret's diameter. We confirmed that **in ECs LPHN2 silencing** results in a statistically significant **2-fold increase** of **vinculin<sup>+</sup>** (**Fig.**



[1F-H](#)) and [paxillin<sup>+</sup> \(Fig. S1F\)](#) FA number and maximum [Feret's diameter](#), which were [rescued by](#) silencing resistant [LPHN2 WT](#), but [not LPHN2 ΔOLF \(Fig. 2A-C and Fig. S2D-F\)](#).

6. *Figure 2D, controls are missing (siCtl with both WT and ΔLOF) and should be displayed at least in supplementary data.*

We usually (*e.g.* [Valdembri et al., 2009, PLoS Biol 7:e25, 10.1371/journal.pbio.1000025](#); [Mana et al., 2016, Nat Commun 7:13546, 10.1038/ncomms13546](#); [Villari et al., 2020, EMBO J., 39:e103661, 10.15252/embj.2019103661](#)) overexpress silencing-resistant cDNA constructs to rescue silenced genes. We do so only in our protein target silenced cells and use as experimental reference the control silenced (siCTL) cells transduced with the lentiviral-deliverable control construct only, because we cannot formally exclude that the transduction of specific cDNAs in siCTL cells would make them prone to potential artifacts due to pleiotropic effects.

7. *Does addition of exogenous FLRT2 elevates Rap1 levels in control cells?*

We found that, while the addition of exogenous FLRT2 rescues the reduction of Rap1-GTP caused by endogenous FLRT2 silencing in stably adherent ECs, it does not further increase the basal levels of Rap1 GTP loading in control cells that express endogenous FLRT2. It is possible that in control stably adherent ECs Rap1 GTP loading is at its plateau (steady state) level. Further work is required to understand if and how modifications in the adhesion state of control ECs may influence their response to exogenous FLRT2 in terms of GTP loading.

8. *Does siFLRT2 changes LPHN2 localization?*

We assessed and found that FLRT2 silencing does not modify LPHN2 subcellular localization.

9. *Figure 5B, colors make the image difficult to analyze.*

We agree with Reviewer's comment and [we have now modified accordingly the color images](#), including a magnification of the region analyzed. The new images are now in [Fig. S3F](#).

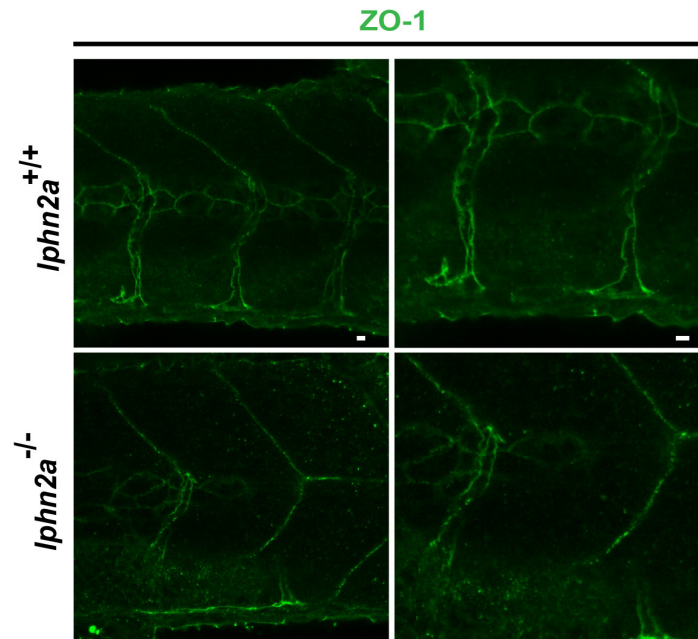
10. *Figure 5C does not exist.*

We apologize for the typo.

11. *In Figure 4C, the tight junction analysis is over interpreted, and images are not convincing as not enough junctions are shown. No quantification is shown to support the description.*

In the [Figure for Reviewer 1](#), we provided pictures of confocal analyses of [whole mount immunostaining of endogenous ZO-1 on both WT and \*lphn2a\* KO](#) zebrafish embryos. Similarly to what observed and carefully quantified [in cultured ECs \(Fig. 5A-B\)](#), while control animals display a linear and continuous recruitment of ZO-1 to intercellular contacts of mCherry<sup>+</sup> ECs of intersomitic blood vessels, [the same cells of \*lphn2a\* KO](#) zebrafish embryos showed a [reduced, discontinuous and fragmented ZO-1](#) staining at the intercellular contacts. The observed abnormal ZO-1 patterning is consistent with our finding in *lphn2a* KO zebrafish embryos: i) disrupted intercellular junctions as evaluated by EM; ii) significantly augmented cancer cell

extravasation; iii) robustly increased basal and VEGF-elicited extravasation of intravascularly injected 70 kDa FITC-Dextran.



Camillo et al., Figure for Reviewer #1

12. In the extravasation study, images don't show clearly what is intra and extra vascular, making the reader not easily convinced by the authors' statements.

We have now performed new extravasation experiments (Fig. 5C) showing vascular permeability defects in *lphn2a* mutants. Microangiography experiments were performed by injecting 70 kDa FITC-Dextran permeabilizing tracer (green) in wild type and *lphn2a* knockout animals. We used vascular endothelial growth factor (VEGF) which is a potent inducer of vascular permeability as a positive control (Weis et al., 2004, *J. Cell. Biol.* 167:223-229). We then quantified the amount of dextran that extravasate in the space outside the vessels as performed by Hoepfner et al. (2012, *Blood* 120:2167-2173) and observed an increased permeability in *lphn2a* KO zebrafish embryos compared to wildtype animals.

**Referee #2:**

1. I had some concerns about the use of a single siRNA for LPHN2, but the key findings are supported by rescues, and the work is validated in a zebrafish model through a different genetic approach.

We did not employ a single siRNA, but a **pool of four different siRNAs (siGENOME SMART pool siLPHN2 M-005651-02, GE Healthcare Dharmacon)**, that is described in the *Materials and Methods* section on [page 15, lines 1-2](#).

Compared to the first version, **rescues with exogenous wild type LPHN2** or the  **$\Delta$ OLF LPHN2 mutant** construct (incapable of binding FLRT2) were employed in the revised manuscript to evaluate the impact of endogenous LPHN2 silencing and its FLRT2 ligand-dependence on a **wide series** of phenotypic features (**Figs. 2, 3, 5, and S2**) namely:

- i) number and **Feret's diameter of ECM adhesions**, as evaluated by **vinculin (Fig. 1F-H and Fig. 2 A-C)** and **paxillin (Fig. S2D-F)** staining;
- ii) number and **size of stress fibers**, as evaluated by **3D STED** super-resolution microscopy along the XZ plane (**Fig. 2D-F**);
- iii) **ECM density-dependent** nuclear translocation of **YAP** and **TAZ (Fig. 1K-L and Fig. 2G-H)**;
- iv) **ECM density-dependent** localization of the tight junction adaptor protein **ZO-1 at intercellular contacts (Fig. 5A-B)**.

2. I think that the morphological changes seen *in vivo* are important. If it was possible to quantify some of the changes shown in Figures 4B and 4C, that would be great. If this is not possible, maybe the authors could reword this section be clear that the observed changes require quantification for certainty. I think this would be enough, as there is quantitative work on the *in vivo* YAP/TAZ outcomes and the extravasation.

We **further characterized** the *in vivo* vascular phenotype. In addition to the previously reported lack of intercellular junctions among ECs and increased cancer cell extravasation, **we quantified and showed** that, compared to wild type animals, *lphn2a* knockout zebrafish embryos display: i) significantly decreased **EC cell surface area**, as evaluated by confocal microscopy Z-sectioning and 3D reconstruction of trunk blood vessels (**Fig. 4E**); ii) evident decreased of **EC area/blood vessel perimeter ratio**, as evaluated in electron microscopy (**Fig. 4F**); iii) a robust increased **extravasation** of intravascularly injected **70 kDa FITC-dextran** both in **basal** condition and also upon stimulation of vascular permeability by treatment with exogenous **VEGF (Fig. 5C)**.

See also rebuttal figure for Referee #1 (Point 11).

3. I wondered if FLRT2 could act *in cis* with LPHN2 on endothelial cells, or if it needed to be shed. And if it could act *in cis*, I wondered if there might be separation of the ligand and receptor in polarised EC monolayers - a bit like the separation of EGFR and its ligand in epithelial cells in airways. That would be interesting in cases where the barrier is disrupted and polarity is temporarily lost.

Based on our finding that, in control cells, LPHN2, but not FLRT2, is highly enriched at ECM adhesion sites, we hypothesized that FLRT2 ligand must be shed to activate at least the large fraction of LPHN2 that concentrates in EC-to-ECM adhesion contacts. However, we also considered that the uncleaved FLRT2 may activate LPHN2 localized outside ECM adhesions, *e.g.* in areas of endothelial cell-to-cell contact. **We discussed this possibility on pages 8, first paragraph**.

*4. And it also seemed that there was potential for communication between neurons and endothelial cells during development, with the neurons presenting an FLRT ligand. That might be observable in movies of the zebrafish - as changes to the elongation of endothelial cells along neurons and/or rates of migration along them.*

We thank Reviewer #2 for such advice. We have been looking at time-lapse movies of the zebrafish during development focusing on a potential communication between neurons and endothelial cells during early development (24hpf to 72hpf). However, we were not able to score any visible changes to the elongation of endothelial cells along neurons and/or rates of migration along them. We therefore believe that more accurate studies (that are however out of the scope of this work) are needed to evaluate whether a potential communication between neurons, presenting a FLRT ligand, and endothelial cells occurs during development.

### Referee #3:

#### Major comments:

1. The authors provide data on the function of LPHN2 in cultured ECs based on the use of a single siRNA. The use of a single siRNA can have problems associated to off-target and non-specific effects. The authors do provide the rescue with haptotaxis, but it would be important to provide a similar rescue with other key functional data (vinculin and stress fibers, YAP assays etc.). Similarly, it would be nice to see recombinant FLRT2 rescuing siFLRT2 in migration assays.

We did not employ a single siRNA, but a **pool of four different siRNAs (siGENOME SMART pool siLPHN2 M-005651-02, GE Healthcare Dharmacon)**, that is described, that are described in the *Materials and Methods* section on [page 15, lines 1-2](#).

Compared to the first version, **rescues with exogenous wild type LPHN2** or the  **$\Delta$ OLF LPHN2 mutant** construct (incapable of binding FLRT2) were employed in the revised manuscript to evaluate the impact of endogenous LPHN2 silencing and its FLRT2 ligand-dependence on a **wide series** of phenotypic features (**Figs. 2, 3, 5, and S2**) namely:

- i) number and **Feret's diameter of ECM adhesions**, as evaluated by **vinculin (Fig. 1F-H and Fig. 2 A-C)** and **paxillin (Fig. S2D-F)** staining;
- ii) number and **size of stress fibers**, as evaluated by **3D STED** super-resolution microscopy along the XZ plane (**Fig. 2D-F**);
- iii) **ECM density-dependent** nuclear translocation of **YAP** and **TAZ (Fig. 1K-L and Fig. 2G-H)**;
- iv) **ECM density-dependent** localization of the tight junction adaptor protein **ZO-1 at intercellular contacts (Fig. 5A-B)** (**see also points 2C and 6**).

Based on our findings that **the key phenotypes** caused by LPHN2 silencing were **rescued by exogenous wild type LPHN2, but not by the  $\Delta$ OLF LPHN2 mutant construct (incapable of binding FLRT2)** (**Fig. 2A-I, Fig. 5B and Fig. S2D-F**), we conclude that in cultured ECs FLRT2-activated LPHN2 inhibits the formation of cell-to-ECM adhesions and actin stress fibers. The lack of FLRT2-elicited LPHN2 signaling results in a significant increase of FAs and actin stress fibers, which in turn cause: i) an increased translocation of YAP/TAZ in the nucleus; ii) a defective recruitment of ZO-1 at intercellular junctions.

*2A. Given the FA- and stress-fiber-inhibitory role of LPHN2, the authors explore whether LPHN2 regulates YAP mechanotransduction. For this, they use hydrogels of intermediate stiffness (5kPa), and show increased nuclear intensity in YAP and TAZ IF, accompanied by increased CTGF expression. Controls are lacking to gauge the effect of 5kPa on YAP - usually, a soft ECM induces the translocation of YAP to the cytoplasm, which is not observed here. This is perhaps related to the general observation that 5kPa is an intermediate situation inducing minor YAP inactivation (see Elosegui-Artola NCB 2016 and Totaro NCOMMS 2017), but it raises some doubts that the soft condition used here is relevant.*

We thank Reviewer #3 for her/his constructive criticisms. Based on previous reports (Birukova et al., 2013, *Microv. Res.*, 87:50–57; Galie et al., 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, Stiffness sensing by cells, 2020, *Physiol. Rev.*, 100:695-724), we repeated the experiments and identified **10 kPa as an optimal stiffness for cultured EC monolayers**. Following the Reviewer suggestions and taking into account previous findings by Pere Roca-Cusachs and collaborators (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548) that **ECM coating density** is a **key determinant of force transmission at integrin-based adhesion sites**, we performed a series of new quantifications to evaluate YAP and TAZ nuclear translocation in both

control and LPHN2 silenced ECs that were also: i) transduced or not with exogenous **wild type or mutant  $\Delta$ OLF LPHN2** constructs; ii) plated on 10 kPa rigid substrates pre-coated with **increasing amounts (1, 3, and 5  $\mu$ g/ml) FN**. In agreement with Elosegui-Artola et al. (*Nat. Cell Biol.*, 2016, 18:540-548) we found that on 10 kPa rigid substrates, increasing amounts (1, 3, and 5  $\mu$ g/ml) of FN resulted in a dose-response translocation of the key mechanosensory effectors YAP/TAZ in the nucleus of control ECs. Furthermore, we found that, the increased number and size of cell-to-ECM adhesions and actin stress fibers correlated with a **higher nuclear translocation of YAP and TAZ** in LPHN2 silenced ECs, compared to control silenced ECs (**Fig. 1K-L**). Of note, such an increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was **rescued by** the introduction of exogenous **wild type LPHN2, but not** by the  **$\Delta$ OLF LPHN2 mutant** construct which is **incapable of binding FLRT2** (**Fig. 2G-H**).

*2B. Please also note on this point that not all GPCR have the same effects: some activate YAP through RHO, some inhibit YAP through PKA (see Guan paper in G&D). Thus, regulation of cAMP might be well in line with a inhibitory GPCR and explain YAP inhibition, independently of FAs and stress-fibers. If so, the relevance of using hydrogels and YAP mechanoresponses becomes less interesting.*

We agree with the reviewer. We were aware of the signaling pathway originally reported by the Guan lab (Yu et al., 2013, *Genes & Dev.* 27:1223-1232), which was reported as reference 106 in the review manuscript by Piccolo and collaborators (Totaro et al., 2018, *Nature Cell Biol.* 20: 888-899) and also cited in our manuscript. In their 2013 paper, Yu and colleagues show how GPCRs can: i) activate YAP/TAZ via  $G\alpha_{12/13}$ , q/11-dependent activation of RHO; ii) inhibit YAP/TAZ via  $G\alpha_s$ /AC/cAMP/PKA-dependent inhibition of RHO. For this reason, as shown in **Fig. S11** and discussed on **page 6 (first paragraph)**, we carefully investigated this aspect and we did not detect any reduction in RHO activation after LPHN2 silencing in ECs.

*2C. In addition, it would also be valuable to understand whether LPHN2 also plays a role in the context of a confluent EC monolayer, or only in the context of isolated motile EC cells, also in light of the fish phenotypes where ECs are fully "confluent".*

Recently, the laboratories of Karl Matter and Maria Balda unveiled a cross-talk between FAs and TJs showing that the **physical properties of the ECM drive** the targeting of the cytoskeletal adaptor **ZO-1 at tight junctions (TJs), thus influencing their assembly** (Haas et al., *Cell Rep.*, 2020, 32: 107924). Therefore, based on previous reports (Birukova et al., *Endothelial barrier disruption and recovery is controlled by substrate stiffness, Microv. Res.*, 2013, 87:50–57; Galie et al., *Application of multiple levels of fluid shear stress to endothelial cells plated on polyacrylamide gels*, 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, *Stiffness sensing by cells*, 2020, *Physiol. Rev.*, 100:695-724), we first identified **10 kPa as an optimal stiffness for cultured EC monolayers**. Next, we plated ECs on 10 kPa rigid substrates pre-coated with **increasing amounts (1, 3, and 5  $\mu$ g/ml) of fibronectin (FN)**. Indeed, Pere Roca-Cusachs and collaborators (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548) first showed that **ECM coating density is a key determinant of force transmission at integrin-based adhesion sites**. In agreement with the mentioned studies by Matter, Balda and Rocha-Cusachs, we observed how increasing amounts of FN result in a **progressive dose-dependent targeting of ZO-1 at endothelial cell-to-cell contacts in control, but not in LPHN2 silenced cells** (**Fig. 5A**). The lack of FN-coating density-dependent ZO-1 translocation caused by the silencing of endogenous LPHN2 was **rescued by** the introduction of exogenous **wild type LPHN2, but not** by the  **$\Delta$ OLF LPHN2 mutant** construct which is incapable of binding FLRT2 (**Fig. 5B**). As mentioned in point 1 above, we conclude that in cultured ECs, LPHN2 inhibits the formation of cell-to-ECM adhesions and actin stress fibers. Indeed, silencing of LPHN2 signaling results in significant increase of FAs and actin stress fibers, which in turn cause: i) an increased translocation of YAP/TAZ in the nucleus; ii) a defective recruitment of ZO-1 at intercellular junctions.

3. *The description of the phenotype in LPHN2aKO embryos does not match the images. Vessels (which vessels? all? in the picture, multiple specific vessels are shown, and is unclear to which vessel the lowest panel refers to, and in which vessel(s) the phenotype is observed) should be thinner and longer, but this is not so apparent from the pictures and no quantification of this phenotype is provided. The same applies to TEM images, where the difference in EC thickness is not so apparent.*

We worked to **better and more precisely describe in the text the phenotype** of *lphn2a* KO zebrafish embryos as documented in images displayed in [Figs. 4, 5, and S3](#). Additionally, we **further characterized** the *in vivo* vascular phenotype. Indeed, together with the previously reported lack of intercellular junctions among ECs and increased cancer cell extravasation, **we quantified and showed** that, compared to wild type animals, *lphn2a* knockout zebrafish embryos display: i) significantly decreased **EC cell surface area**, as evaluated by confocal microscopy Z-sectioning and 3D reconstruction of trunk blood vessels ([Fig. 4E](#)); ii) evident decreased **EC area/blood vessel perimeter ratio**, as evaluated in electron microscopy ([Fig. 4F](#)); iii) a robust increased **extravasation** of intravascularly injected **70 kDa FITC-Dextran** both in **basal** condition and also upon stimulation of vascular permeability by treatment with exogenous **VEGF** ([Fig. 5C](#)).

See also rebuttal Figure for Referee 1 (Point 11).

4. *Data in embryos suggest a role for LPHN2a in vessel formation and in regulation of Hippo. However, whether the two are causally related (as suggested by the authors) or just two associated events (for example, loss of cell-cell adhesions might be driving Hippo signalling) remains unknown. Please revise the claims and discussion accordingly.*

We agree with the Reviewer and revised claims and discussion as (s)he requested. In general, as also mentioned in points 1 and 2C above, we would favor and propose a working model in which FLRT2-activated LPHN2 inhibits the formation of cell-to-ECM adhesions and actin stress fibers, while promoting the assembly of TJs in ECs. While **TJs inhibit** the nuclear translocation of **YAP and TAZ** through their Hippo pathway-dependent phosphorylation, **FAs** and the associated F-actin **stress fibers** exert exactly the opposite effect **promoting YAP/TAZ** nuclear localization and transcriptional function (Karaman and Halder, 2018, *Cold Spring Harb. Perspect. Biol.*, 10:a028753; Moya & Halder, 2019, *Nature Rev. Mol. Cell Biol.*, 20: 211–226). As discussed on [page 12](#), we then posit that the nuclear translocation and functional **activation of YAP/TAZ**, observed upon LPHN2 silencing/knockdown *in vitro* ([Fig. 1K-M](#)) and *in vivo* ([Fig. 4C-D](#)), lie **downstream of** the increased formation of **FAs/stress fibers** and the disassembly of **TJs**. Of note, such an increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was **rescued by** the introduction of exogenous **wild type LPHN2, but not by the ΔOLF LPHN2 mutant** construct which is incapable of binding FLRT2 ([Fig. 2G-H](#)).

5. *Data on enhanced vessel permeability are nice, but need to be complemented by a more standard dextran injection. If TJ are directly affected (as opposed to the ability of ECs to interact with metastatic cells, for example), dextran should be leaky.*

As suggested by the Reviewer, we **further characterized** the *in vivo* vascular phenotype. In addition to the previously reported lack of intercellular junctions among ECs and increased cancer cell extravasation, **we quantified and showed** that, compared to wild type animals, *lphn2a* knockout zebrafish embryos display: i) significantly decreased **EC cell surface area**, as evaluated by confocal microscopy Z-sectioning and 3D reconstruction of trunk blood vessels ([Fig. 4E](#)); ii) evident decreased **EC area/blood vessel perimeter ratio**, as evaluated in electron microscopy ([Fig. 4F](#)); iii) a robust increased **extravasation** of intravascularly injected **70 kDa FITC-dextran** both in **basal** condition and also upon stimulation of vascular permeability by treatment with exogenous **VEGF** ([Fig. 5C](#)).

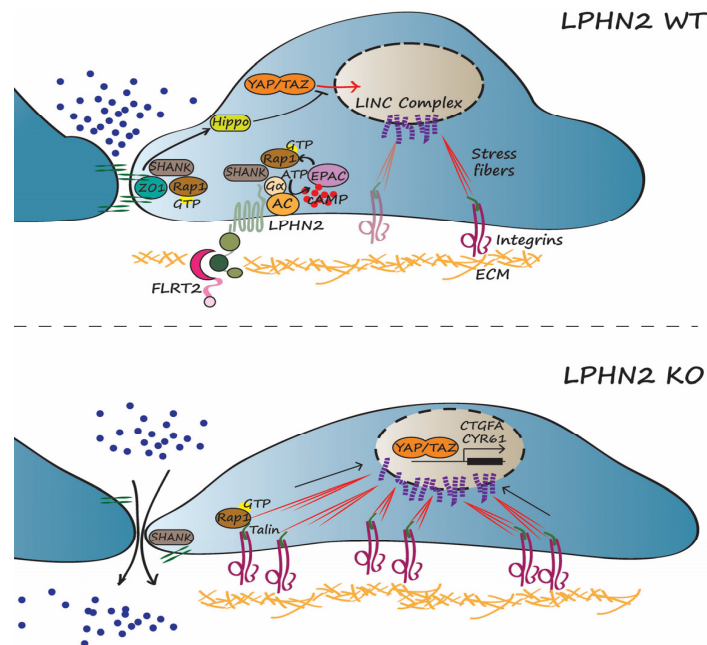
In the [Figure for Reviewer 1](#), we provided pictures of confocal analyses of **whole mount immunostaining of endogenous ZO-1 on both WT and *lphn2a* KO** zebrafish embryos. Similarly to what

observed and carefully quantified in cultured ECs (Fig. 5A-B), while control animals display a linear and continuous recruitment of ZO-1 to intercellular contacts of mCherry<sup>+</sup> ECs of intersomitic blood vessels, **the same cells of *lphn2a* KO zebrafish embryos showed a reduced, discontinuous and fragmented ZO-1 staining** at the intercellular contacts. The observed abnormal ZO-1 patterning is consistent with our finding in *lphn2a* KO zebrafish embryos disrupted intercellular junctions, significantly augmented cancer cell extravasation, and robustly increased basal and VEGF-elicited extravasation of intravascularly injected 70 kDa FITC-Dextran.

6. It remains unclear how the proposed function of LPHN2 in regulating EC-to-ECM adhesions (shown *in vitro*) relates to the *in vivo* TJ phenotype. Is this also observed *in vitro*, with confluent monolayers (e.g. transendothelial conductance assays, or fluorescent dextran permeability)? Is this dependent on cAMP and RAP1 signaling? and/or on Hippo?

We agree with the Reviewer. Thanks to recently published studies, we can now more robustly discuss and provide a clearer link between LPHN2 signaling, cell-to-ECM adhesions and TJs.

We showed that **LPHN2 promotes the GTP-loading and activation of the small GTPase Rap1**, which is a well-known regulator of **cell-to-ECM adhesions** (Larrigue et al., 2016, *Blood*, 128:479-487; Colò et al., 2012, *J. Cell Sci.* 125: 5338-5352). While known for its ability to signal and increase **EC barrier function** (Bos, 2018, *Cold Spring Harb. Perspect. Med.*, 8:a031468), Rap1 was only very recently formally showed to promote the **formation of tight junctions** (TJs; Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088), which in ECs play a crucial role in the control of vascular permeability.



Therefore, it is **conceivable that LPHN2 activation of Rap1 acts both to inhibit** the formation of focal adhesions (FAs) and **to promote** the assembly of TJs, which increase EC barrier function. Further work is required to thoroughly understand the mechanisms by which LPHN2 exerts, *via* Rap1, its opposite effect on FAs and TJs. However, as discussed in the last part of our revised manuscript (and depicted in the *schematic* above), **the ability of LPHN2 to directly bind** the central PSD-95/Dlg/ZO-1 (PDZ) domain and multiple ankyrin repeat domains (SHANK) proteins is in this regard likely crucial. Indeed, the binding of the N-terminal Shank/ProSAP N-terminal (SPN) domain of SHANK to Rap1-GTP was found both to: i) **suppress talin**-mediated integrin activation and FA development (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305); ii) **promote** the assembly of TJs (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088). Therefore, LPHN2 may favor the turnover of FAs and the assembly of TJs by funneling Rap1-GTP towards



SHANK.

Concerning YAP/TAZ, while **TJs inhibit** the nuclear translocation of **YAP and TAZ** through their Hippo pathway-dependent phosphorylation, **FAs** and the associated F-actin **stress fibers** exert exactly the opposite effect **promoting YAP/TAZ** nuclear localization and transcriptional function (Karaman and Halder, 2018, *Cold Spring Harb. Perspect. Biol.*, 10:a028753; Moya & Halder, 2019, *Nature Rev. Mol. Cell Biol.*, 20: 211–226). As discussed on [page 12](#), we then posit that the nuclear translocation and functional **activation of YAP/TAZ**, observed upon LPHN2 silencing/knockdown *in vitro* ([Fig. 1K-M](#)) and *in vivo* ([Fig. 4C-D](#)), lie **downstream** of the increased formation of **FAs/stress fibers** and the disassembly of **TJs**. Of note, such an increased YAP/TAZ translocation caused by the lack of endogenous LPHN2 was **rescued by** the introduction of exogenous **wild type LPHN2, but not** by the  **$\Delta$ OLF LPHN2 mutant** construct which is incapable of binding FLRT2 ([Fig. 2G-H](#)).

For the aspect concerning the crosstalk between FAs and TJs, please refer to point 2C.

*Minor points:*

1. Please describe better the haptotaxis assay - it is not clear why the authors state "migration towards collagen", when it is my understanding what is being measured is the displacement on collagen-coated dishes. Accordingly, provide a better definition of the y-axis in the migration assays (what does relative means? relative to the initial position? what is the measure, microns? is the measure the total path, or the radius from the initial position?). In the text, the same assays also described as "directional migration" (figure 3) but the assay is the same, and it is not clear why this assay is particularly instructive on the directionality. Is this measuring migration persistence? Please clarify and use terms accordingly. This also related to the use of "chemorepulsion" - it is unclear whether the phenotypes are just related to migration speed (as the reviewer understands), or to migration directionality (which would imply some kind of asymmetric source of FLRTs?).

[On pages 18-19](#), we provided additional information on the employed impedance-based haptotactic migration system produced by ACEA/Agilent Technologies. Moreover, axis labels were carefully checked.

2. When introducing FLRT as possible ligands of LPHN2, this comes unexpected because in the introduction LPHN2 are described as ECM receptors. Please describe better in the introduction.

Compatibly with text limits of JCB Report manuscript format, we provided additional background on LPHN2 and FLRT2 in the *Introduction* section ([Page 4](#)).

3. Please describe better in the text the "ligand-receptor *in situ* binding assay" by indicating the use of recombinant FLRT2.

[On pages 17-18](#), we provided additional information on the ligand-receptor *in situ* binding assay.

4A. When describing LPHN2 expression in zebrafish, it is said it is enriched in vascular ECs of the DA and PCV, but compared to what? to the surrounding tissues, or to other EC? In other words, is LPHN2 expressed mainly in these vessels and not in others, or in all ECs).

We performed a **new** series of stainings with **anti-LPHN2** antibodies followed by **confocal fluorescence microscopy** analyses on transversal sections of **wild type** and ***lphn2a* knock-out** zebrafish embryos with ***Tg(kdrl:EGFP)<sup>S843</sup>*** genetic background in which EGFP is selectively expressed in ECs. In [Fig. 4A](#), we show that **LPHN2** is expressed in EGFP<sup>+</sup> ECs of both dorsal aorta (**DA**) and pericardinal vein (**PCV**) of wild type (arrows),

but not *lphn2a* knock-out zebrafish embryos. Furthermore, **high magnification areas** are shown. Overall, it is evident that LPHN2 is expressed mainly by endothelial cells at these stages of development. We did not perform a comparative study looking at other tissues (*e.g.* nervous tissues) throughout development, though.

*4B. Technically, is the antibody epitope conserved?*

As described in the Materials and Methods section the 2Y4824 rabbit polyclonal antibody (pAb) anti-LPHN2 was produced for us by Eurogentec by immunizing animals with peptide GGKTDIDLAVDENGL (amino acids 259-274). **This peptide of human LPHN2 is fully conserved** in the orthologous proteins of *Danio rerio* and *Mus musculus*.

*4C. Is the staining lost in LPHN2a KO?*

**Yes, it is lost.** We performed a **new** series of stainings with **anti-LPHN2** antibodies followed by **confocal fluorescence microscopy** analyses on transversal sections of **wild type** and ***lphn2a* knock-out** zebrafish embryos with ***Tg(kdrl:EGFP)<sup>s843</sup>*** genetic background in which EGFP is selectively expressed in ECs. In **Fig. 4A**, we show that **LPHN2** is expressed in EGFP<sup>+</sup> ECs of both dorsal aorta (**DA**) and pericardinal vein (**PCV**) of wild type, but not *lphn2a* knock-out zebrafish embryos. Furthermore, **high magnification areas** are shown.

*5. Please briefly introduce what the *Tg(kdrl:EGFP)<sup>s843</sup>* line is used for, for the sake of non-experts.*

The transgenic (Tg) *Tg(kdrl:EGFP)<sup>s843</sup>* line is a zebrafish transgenic line harboring the EGFP protein under the control of the zebrafish VEGFR2 (kdrl) promoter. It is a Tg line widely used by the zebrafish community to mark endothelial throughout development for vascular studies in zebrafish (<https://zfin.org/ZDB-ALT-050916-14>).

We added a description in the text on [page 20](#).

*6. Is the choice of LPHN2a mutagenesis dictated by chance (vs. possible LPHN2b, c, d etc)? In other words, is there room for functional redundancy with other duplicated genes, so that one may expect stronger phenotypes by complete inactivation of LPHN2s? This should be discussed.*

At the best of our knowledge, other than *lphn2a* (*adgrl2a*), a second gene only, namely *lphn2b.1* (*agdr12b.1*; Gene ID: 110437953 ; ZFIN ID: ZDB-GENE-130116-4 ; Ensembl: ENSDARG00000111712), exists in *Danio rerio*. *Lphn2b.1/Agdr12b.1* is a very short version (534 amino acids) of *Lphn2a* that contains only the N-terminal region, which contains the olfactomedin domain, responsible for binding to the FRLT2 ligand. The remaining extracellular, transmembrane and cytosolic portions are missing. Since it is lacking the signaling domain, it is difficult to hypothesize that *Lphn2b.1/Agdr12b.1* could compensate for the lack of *lphn2a*. One possibility may be that *Lphn2b.1/Agdr12b.1* acts in physiological conditions as a natural decoy/dominant negative protein for *Lphn2a*.

*7. Why ECs are not described with their more common name HUVECs?*

For sake of simplicity, above all when a study is performed both *in vitro* on human ECs and *in vivo* in zebrafish embryos, we are in general describing ECs as such and detailing their origin only in the *Materials and Methods* section.

8. Please provide the complete siRNA sequences in the methods including controls if possible.

As described in the *Materials and Methods* section on [page 15, lines 1-2](#), to silence LPHN2 and FLRT2 in human ECs we employed siGENOME SMART pool siLPHN2 M-005651-02 and siGENOME SMART pool siFLRT2 M-009104-00 from GE Healthcare Dharmacon. siGENOME SMART pools are patented combinations of 4 different gene specific and pre-validated siRNAs, whose sequence is disclosed by GE Healthcare Dharmacon to the buyer, who is not allowed to share it publicly. The same applies to siGENOME Non-Targeting siRNA #1 control.

May 7, 2021

Re: JCB manuscript #202006033R

Prof. Guido Serini  
University of Turin  
Department of Oncology  
Strada Provinciale 142 Km 3.95  
Candiolo 10060  
Italy

Dear Dr. Serini,

Thank you for submitting your revised manuscript entitled "LPHN2 inhibits vascular permeability by differential control of endothelial cell adhesion." The manuscript has been seen by the original reviewers whose full comments are appended below. While we believe that the work is suitable for JCB, some important issues remain that must be addressed prior to publication.

You will see that Reviewer #1 feels that some of your new data regarding LPHN2 effects on YAP/TAZ activation and cell junction defects in vivo are not convincing and not directly comparable with in vitro experiments. We believe these concerns are important to address with new experiments and/or textual revisions of the current conclusions, particularly points #2,3,4,7. We also concur that your assay would be better described as a migration assay rather than a haptotaxis assay. The question of whether LPHN2-mediated signaling effects on ZO-1 are linked to LPHN2 effects on FAs and/or YAP/TAZ activation is interesting but in our view this is outside the scope of the present study. Reviewer #3 has two points that are also important and should be fairly easy to address with text revisions. One of these is regarding the sequences of your siRNAs. Please note that JCB policy requires reporting of all siRNA sequences. Including these is an essential prerequisite for publication and we cannot consider a revision which lacks this information.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. The typical time frame for such final revisions is one month. However, we understand that measures implemented to limit the spread of COVID-19 also pose challenges to scientific researchers so please let us know if you will require more time. Please note that we expect to make a final decision without additional reviewer input upon resubmission. Along with the revised manuscript please submit a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Johanna Ivaska, PhD  
Monitoring Editor

Dan Simon, PhD  
Scientific Editor  
Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The reviewer acknowledges the great effort made by the authors to improve the previous version of their manuscript. The authors performed several additional experiments and they have addressed many of the original points. However, there are still major concerns (new or remaining) that required to be address prior to publication.

1- The assay authors used is not an haptotaxis assays. Haptotaxis is the migration towards a gradient of adhesion molecules. However, as far as the reviewer could understand, this assay is a modified Boyden chamber invasion assay in which authors change the type of coating of the upper chamber. If the assay would be solely based on haptotaxis, cells would not invade the bottom chamber. Moreover, it is not because the assay was published as an haptotaxis assay in other journals that makes this assay an haptotaxis assay.

In the material and methods, authors referred to a CIM-Plate 16 (which stand for cell invasion and migration, which is the normal Boyden chamber assay). Any gradient would be determined by differences in the composition in media of the upper and lower chambers. Thus, the reviewer would like the authors to correct any mention to haptotaxis. Having said this, the reviewer is not challenging the role of LPHN2 in cell migration, rather that it is involved in haptotaxis.

2- In Figure 4 - zebrafish experiments showed increased CTGF promoter activation (used as a reporter for YAP/TAZ activation) in endothelial cells. 3D segmentation and images showed a strong activation in cells at the pericardinal vein but very low in aorta (fig.4C), however analysis of endothelial cell area/vessel diameter (fig.4E-F) was performed in arterial cells. This is inconsistent and questions the relationship between YAP/TAZ levels, LPHN2 function and cell shape/size changes. Moreover, cell size/shape analysis was not a measure analysed in vitro, thus any possible link between in vitro discoveries on FAs and YAP activity and zebrafish data is very speculative.

3- Authors wrote pag.9 "Quantitative confocal microscopy analyses showed that ECs forming the trunk vasculature were much thinner and stretched along the main blood vessel axis, resulting in a decreased EC size in *lphn2a*<sup>-/-</sup> compared to wild type embryos (Fig. 4E)". However, the quantification is not convincing. The Tg(*kdr*:EGFP) fish line cannot be used to perform such quantification, as cell boundaries between endothelial cells cannot be established. This would require the usage of a marker of cell junctions, or, to be even more accurate, the clonal expression of a membrane-bound fluorophore.

Fig.4F does show some apparent thinning of the endothelium. However, the link between endothelial cell thinning, FA and/or TJ formation/assembly/stability is uncertain. Thus, the links between different phenotypes (in vitro and in vivo) are weak and they are not easy to interpret.

4- TEM analysis of EC junctions in Fig. 4G is not convincing. The images that authors provide are not of sufficient quality to support the statement "revealed the lack of properly organized TJs (red arrow in Fig. 4G)". For instance, how authors can distinguish between adherens junctions from tight

junctions? Why is not possible to observe a lumen in the micrograph of the KO animal? Moreover, authors provide further images in the response to reviewers using ZO-1 staining. Why these data are not included in the current version of the manuscript?

5- The link between FA and TJ, and their relationship with LPHN2, are even more unclear if one takes all the data together. Increased adhesion to ECM seems to have a positive effect on assembly of TJ and YAP signalling, demonstrated by the increased stability/formation of TJ (Fig.5A) and increased nuclear YAP/TAZ (Fig.2H) when cells are plated in higher concentrations of fibronectin. Loss of LPHN2 leads to increased adhesion (more FAs) and more YAP/TAZ. So, one would expect an increase in ZO-1 assembly in siLPHN2, whilst the authors see the opposite. Thus, it is currently unclear if the positive effects of LPHN2-mediated signalling on ZO-1 localization are associated with its negative effects on FAs and/or YAP/TAZ activation. Authors would need to manipulate FAs or YAP/TAZ alone and confirm its effects on ZO-1 localisation and TJ-assembly in their assay.

Thus, the overall conclusion that "As substantiated in cultured ECs, LPHN2 favors the mechanochemical crosstalk by which FA-sensed ECM stiffness and density promote the formation of TJs", is not supported by the data.

Minor concerns:

6- In Figure 2A/G - siControl and siLPHN2 cells showed GFP signals, however there is no indication of any transfection. Please clarify. Also, can authors explain why cells evidence strong nuclear signal, in striking contrast to Figure 1B. Can authors explain.

7- The authors do not show quantification for a normal recruitment of VE-cadherin to cell-cell boundaries. The authors only display one representative image of each condition (Fig.5A,B). This is important for 2 reasons: 1) the reviewer has the impression that VE-cadherin staining intensity and distribution increases with increased amounts of fibronectin and decreases in siLPHN2; 2) the levels of VE-cadherin were used to quantify the impact of matrix and loss of LPHN2 in ZO-1 distribution (fig.5A-right panel). Moreover, which was the fibronectin concentration used in experiments for Fig.5B. The reviewer could not retrieve the information.

Reviewer #2 (Comments to the Authors (Required)):

I liked this manuscript at first review and had only some minor requests and suggestions. All of these have been met in review and the revised paper is improved by the review process.

Reviewer #3 (Comments to the Authors (Required)):

Overall, the authors provide a satisfactory revision of their manuscript, which is now stronger. There are two remaining issues that need to be fixed by correcting the text before publication.

First, the authors now disclose they used a Dharmacon SMART pool (a mix of four different siRNAs), and provide more data in support of the specificity of their reagents by using rescue assays with WT vs mutant LPHN2, which is convincing. However, they do not provide the sequence of the siRNAs, claiming Dharmacon does not allow such disclosure. This is not true, because the authors are given the sequences when they purchase the pools, and they are completely free to

disclose this information at the moment of publication. According to the Dharmacon website, limitations only apply to whole siRNA libraries. This point is important in light of reproducibility - the manuscript heavily relies on these reagents, and someone may want in future to repeat those data by using exactly the same siRNA pools (and not, for example, a renewed mix of unknown specificity from Dharmacon itself). On this point, the authors should also state whether the cDNAs used for rescue were of WT sequence (and, thus, the rescue was an effect of expression over the efficiency of the siRNA) or if they were mutagenized to obtain an siRNA-resistant mRNA, and how.

Second, the question of hydrogel stiffness has not been addressed. The authors claim that LPHN2 regulates YAP mechanosignalling, starting from the notion that LPHN2 regulates stress fibers and focal adhesion dynamics, and on the notion that these structures are important for mechanosignalling in general. However, they used hydrogels that are very stiff (in the revised version, even stiffer than in the first), on which YAP is not inhibited. In other words, they do not show that a very soft hydrogel induces YAP inhibition (compared to a stiffer one), and that in those conditions siLPHN2 is sufficient to reactivate YAP. So, the effect of LPHN2 is a basal one, occurring independently of stiffness. Similarly, they did not provide data on LPHN2 regulating YAP localization in confluent monolayers, which would have provided alternative/additional data to gauge this point. Finally, the fact that LPHN2 is a GPCR acting on cAMP levels and thus likely on PKA indicates it could well regulate the Hippo pathway directly, which has not been addressed experimentally. In absence of data making any specific experimental connection with a mechanical perturbation of ECs, and considering that the effects on YAP might be well due (also? mainly?) to the crosstalk with TJs and/or to a GPCR signaling function, the authors should tone down their claims on mechanosignaling, and focus more in general on Hippo signaling.

### Referee #1:

#### Major points:

1. The assay authors used is not an haptotaxis assays. Haptotaxis is the migration towards a gradient of adhesion molecules. However, as far as the reviewer could understand, this assay is a modified Boyden chamber invasion assay in which authors change the type of coating of the upper chamber. If the assay would be solely based on haptotaxis, cells would not invade the bottom chamber. Moreover, it is not because the assay was published as an haptotaxis assay in other journals that makes this assay an haptotaxis assay. In the material and methods, authors referred to a CIM-Plate 16 (which stand for cell invasion and migration, which is the normal Boyden chamber assay). Any gradient would be determined by differences in the composition in media of the upper and lower chambers. Thus, the reviewer would like the authors to correct any mention to haptotaxis. Having said this, the reviewer is not challenging the role of LPHN2 in cell migration, rather that it is involved in haptotaxis.

We now describe, throughout the whole manuscript, our ECM-elicited endothelial cell (EC) directional motility assay as a **migration assay** rather than a haptotaxis assay.

2. In Figure 4 – zebrafish experiments showed increased CTGF promoter activation (used as a reporter for YAP/TAZ activation) in endothelial cells. 3D segmentation and images showed a strong activation in cells at the pericardinal vein but very low in aorta (fig.4C), however analysis of endothelial cell area/vessel diameter (fig.4EF) was performed in arterial cells. This is inconsistent and questions the relationship between YAP/TAZ levels, LPHN2 function and cell shape/size changes. Moreover, cell size/shape analysis was not a measure analysed *in vitro*, thus any possible link between *in vitro* discoveries on Fas and YAP activity and zebrafish data is very speculative.

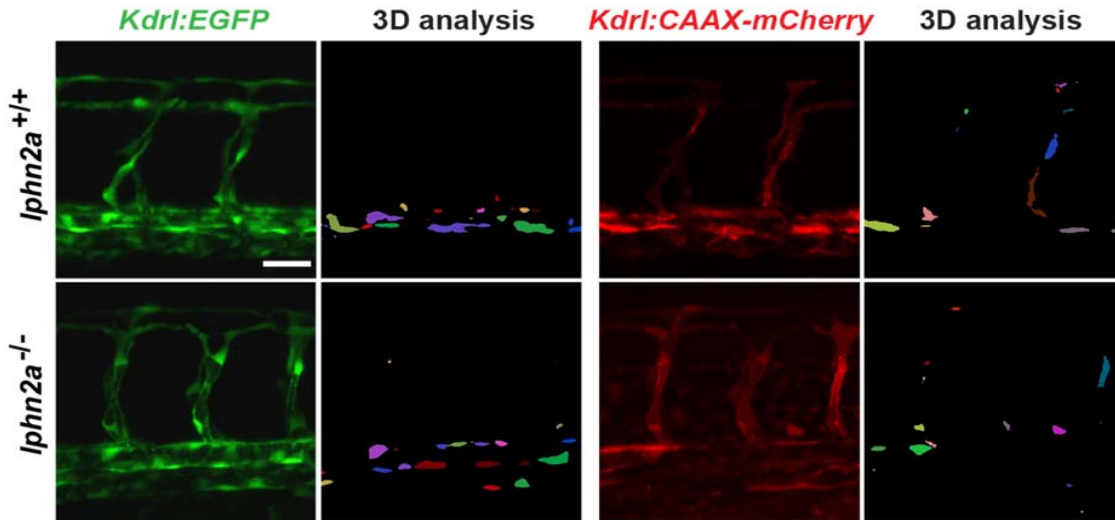
We thank the Reviewer for having pointed out that the images in Fig. 4C displayed a stronger YAP/TAZ activation reporter signal is stronger in ECs of the pericardinal vein (PCV) compared to dorsal aorta (DA). As we previously published in Astone et al. (*Sci. Rep.* 8, 10189, 2018; <https://doi.org/10.1038/s41598-018-27657-x>), the YAP/TAZ activation reporter signal is detected in ECs without expression differences between arteries and veins. Therefore, to avoid misunderstanding and **to consistently document** the relationship between YAP/TAZ levels and LPHN2 function **in zebrafish ECs of the trunk vessels**, we further characterized CTGF promoter activation in ECs of the trunk vasculature, namely DA, PCV, and intersomitic blood vessels (ISV). To this aim, we imaged embryos between 56 to 72 hpf, time points in which we found equal **reporter signal in DA and PCV** that allowed us to better quantify YAP/TAZ activation in trunk ECs. We found that the reporter activity (measured as CTGF promoter-driven mCherry expression) is **significantly higher in trunk ECs of *lphn2a* knockout zebrafish embryos compared to wild type animals (new Fig. 4C)**. These data convincingly indicate the **lack of *lphn2a*** causes a general **upregulation of YAP/TAZ activation in ECs regardless of the vein/arterial nature** of the blood vessels.

3. Authors wrote pag.9 "Quantitative confocal microscopy analyses showed that ECs forming the trunk vasculature were much thinner and stretched along the main blood vessel axis, resulting in a decreased EC size in *lphn2a*<sup>-/-</sup> compared to wild type embryos (Fig. 4E)". However, the quantification is not convincing. The *Tg(kdrl:EGFP)* fish line cannot be used to perform such quantification, as cell boundaries between endothelial cells cannot be established. This would require the usage of a marker of cell junctions, or, to be even more accurate, the clonal expression of a membrane-bound fluorophore. Fig.4F does show some apparent thinning of the endothelium. However, the link between endothelial cell thinning, FA and/or TJ formation/assembly/stability is uncertain. Thus, the links between different phenotypes (*in vitro* and *in vivo*) are weak and they are not easy to interpret.

Following the suggestion of the Reviewer, with the aim to better characterizing cell boundaries between ECs, we employed the *Tg(kdrl:CAAX-mCherry)<sup>uto2</sup>* fish line in which **ECs express membrane-bound CAAX-mCherry**. However, as shown in the **representative image below**, **cytosolic GFP is much more effective** than



membrane bound CAAX-mCherry in [distinguishing cell boundaries](#) between adjacent ECs of trunk large blood vessels such as DA (see the [figure below here](#)).



4. TEM analysis of EC junctions in Fig. 4G is not convincing. The images that authors provide are not of sufficient quality to support the statement "revealed the lack of properly organized TJs (red arrow in Fig. 4G)". For instance, how authors can distinguish between adherens junctions from tight junctions? Why is not possible to observe a lumen in the micrograph of the KO animal? Moreover, authors provide further images in the response to reviewers using ZO-1 staining. Why these data are not included in the current version of the manuscript?

We agree with the reviewer and we have now removed the TEM panels of previous Fig. 4G.

Since it is known (see Dejana, 2004, *Nat. Rev. Mol. Cell Biol.*, 5:261-270, Fig. 1 in particular) that *in vivo* ECs, in contrast to epithelial cells, display less rigidly organized and frequently really complex interendothelial cell-cell contacts (with a significant amount of overlap between adjacent ECs seen by transmission-electron-microscopy - TEM), confocal microscopy on immunofluorescence-stained blood vessels is required to properly analyze the *in vivo* organization of EC TJs. As requested, we included pictures of confocal analyses of whole mount immunostaining of endogenous ZO-1 on both WT and *Iphn2a* KO zebrafish embryos (new Fig. 4G). While control animals display a linear and continuous recruitment of ZO-1 to intercellular contacts of intersomitic blood vessel ECs, the same cells of *Iphn2a* KO zebrafish embryos show a reduced, discontinuous and fragmented ZO-1 staining at the intercellular contacts.

Finally, we apologize, we forgot to label ECs and lumens in Fig. 4F of the revised manuscript. ECs and lumens are now indicated with lettering, as "EC" and "L" respectively.

5. The link between FA and TJ, and their relationship with LPHN2, are even more unclear if one takes all the data together. Increased adhesion to ECM seems to have a positive effect on assembly of TJ and YAP signalling, demonstrated by the increased stability/formation of TJ (Fig.5A) and increased nuclear YAP/TAZ (Fig.2H) when cells are plated in higher concentrations of fibronectin. Loss of LPHN2 leads to increased adhesion (more FAs) and more YAP/TAZ. So, one would expect an increase in ZO-1 assembly in siLPHN2, whilst the authors see the opposite. Thus, it is currently unclear if the positive effects of LPHN2-mediated signalling on ZO-1 localization are associated with its negative effects on FAs and/or YAP/TAZ activation. Authors would need to manipulate FAs or YAP/TAZ alone and confirm its effects on ZO-1 localisation and TJ assembly in their assay. Thus, the overall conclusion that "As substantiated in cultured ECs, LPHN2 favors the mechanochemical

*cross-talk by which FA-sensed ECM stiffness and density promote the formation of TJs", is not supported by the data.*

We showed that **LPHN2 promotes the GTP-loading and activation of the small GTPase Rap1**, which is a well-known regulator of **cell-to-ECM adhesions** (Larrigue et al., 2016, *Blood*, 128:479-487; Colò et al., 2012, *J. Cell Sci.* 125: 5338-5352). While known for its ability to signal and increase **EC barrier function** (Bos, 2018, *Cold Spring Harb. Perspect. Med.*, 8:a031468), **Rap1** was only very recently formally showed to **promote the formation of tight junctions** (TJs; Sasaki et al., 2020, *Cell Rep.* 31:107407), which in ECs play a crucial role in the control of vascular permeability.

It is hence **conceivable that LPHN2 activation of Rap1 acts both to inhibit** the formation of focal adhesions (**FAs**) and **to promote** the assembly of **TJs**, which increase EC barrier function. In this regard, **the ability of LPHN2 to directly bind** the central PSD-95/Dlg/ZO-1 (**PDZ**) domain of the SH3 and multiple ankyrin repeat domains (**SHANK**) proteins is likely crucial. Indeed, the binding of the N-terminal Shank/ProSAP N-terminal (**SPN**) domain of **SHANK** to **Rap1-GTP** was found both to: i) **suppress talin-mediated integrin activation** and FA development (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305); ii) **promote** the assembly of **TJs** (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088).

Therefore, LPHN2 may favor the turnover of FAs and the assembly of TJs by funneling Rap1-GTP towards SHANK. We formally investigated the latter hypothesis and **found that in cultured ECs LPHN2 silencing significantly reduces the physical interaction between the small GTPase Rap1 and SHANK2** (**new Fig. S2I**).

*Minor points:*

*6. In Figure 2A/G - siControl and siLPHN2 cells showed GFP signals, however there is no indication of any transfection. Please clarify. Also, can authors explain why cells evidence strong nuclear signal, in striking contrast to Figure 1B. Can authors explain.*

As previously detailed in *Material & Methods* (**page 13, first para**) and legend of **Fig. 2**, cells were first transduced with **GFP-containing pCCL.sin.cPPT.polyA.CTE.eGFP.minhCMV.hPGK.Wpre (pCCL) lentivirus** carrying or not silencing-resistant mouse WT or  $\Delta$ OLF Lphn2, and then oligofected with either siCTL or siLPHN2 siRNAs. In this case, GFP is not fused to any protein and it is employed as a **marker of effective lentiviral transduction** of the observed cells.

In contrast, as previously specified in the text (**page 5, first para**), “to verify the relationships of both LPHN2 subunits with ECM adhesions, we transfected ECs with an N-terminally HA-tagged and C-terminally EGFP-tagged mouse Lphn2 construct (**HA-Lphn2-EGFP**; Fig. S1B). We found that, similarly to endogenous LPHN2, both the extracellular and the intracellular moieties of HA-Lphn2-EGFP co-localized with vinculin at cell-to-ECM adhesion sites (Fig. 1B).”, **GFP is C-terminally fused in frame with mouse Lphn2** to allow the simultaneous visualization by confocal microscopy of the **N-terminal (HA-tagged) and C-terminal (GFP-tagged) halves of Lphn2 physiologically cut by proteases**. The observed colocalization in adhesion sites of the HA-N-terminal and C-terminal-GFP halves of Lphn2 are consistent with their previously reported non-covalent association (Langenhan et al., 2013, *Sci. Signal.* 6:re3).

*7. The authors do not show quantification for a normal recruitment of VE-cadherin to cell-cell boundaries. The authors only display one representative image of each condition (Fig. 5A,B). This is important for 2 reasons: 1) the reviewer has the impression that VE-cadherin staining intensity and distribution increases with increased amounts of fibronectin and decreases in siLPHN2; 2) the levels of VE-cadherin were used to quantify the impact of matrix and loss of LPHN2 in ZO-1 distribution (fig.5A-right panel). Moreover, which was the fibronectin concentration used in experiments for Fig.5B. The reviewer could not retrieve the information*

We quantified the recruitment of VE-cadherin to intercellular contacts and did not observe any significant difference neither after plating ECs on increasing amounts of fibronectin ([new Fig. S1L](#)) nor in LPHN2-silenced ECs compared to controls ([new Fig. S1M](#)). This is the reason why we employed VE-cadherin as an intercellular junction reference to normalize the amounts of ZO-1.

We apologize for having not specified the fibronectin concentration used in experiments for [Fig. 5B](#), which is now indicated (5 µg/ml).

**Referee #2:**

*I liked this manuscript at first review and had only some minor requests and suggestions. All of these have been met in review and the revised paper is improved by the review process.*

### Referee #3:

Overall, the authors provide a satisfactory revision of their manuscript, which is now stronger. There are two remaining issues that need to be fixed by correcting the text before publication.

1. First, the authors now disclose they used a Dharmacon SMART pool (a mix of four different siRNAs), and provide more data in support of the specificity of their reagents by using rescue assays with WT vs mutant LPHN2, which is convincing. However, they do not provide the sequence of the siRNAs, claiming Dharmacon does not allow such disclosure. This is not true, because the authors are given the sequences when they purchase the pools, and they are completely free to disclose this information at the moment of publication. According to the Dharmacon website, limitations only apply to whole siRNA libraries. This point is important in light of reproducibility - the manuscript heavily relies on these reagents, and someone may want in future to repeat those data by using exactly the same siRNA pools (and not, for example, a renewed mix of unknown specificity from Dharmacon itself). On this point, the authors should also state whether the cDNAs used for rescue were of WT sequence (and, thus, the rescue was an effect of expression over the efficiency of the siRNA) or if they were mutagenized to obtain an siRNA-resistant mRNA, and how.

We apologize. **We incorrectly interpreted the confidentiality statement of Dharmacon** about their siRNAs (“Buyer agrees that technical information that Dharmacon provides to Buyer is the confidential and proprietary information of Dharmacon. Buyer agrees to (i) keep such information confidential and not disclose such information to any third party, and (ii) use such information solely for Buyer’s internal purposes”). After Dharmacon formal consent, **we now provide**, in the *Materials and Methods* section ([page 16, first para](#)), **the sequences of the four siRNAs mixed in the siGENOME SMART pool of both siLPHN2 and siFLRT2** that we employed in our *in vitro* studies.

Concerning the **cDNAs used for rescue**, as we stated in the previous versions of our manuscript (e.g., page 5, last para; legend of Figs. 1E, 2A, and S2D-F), in human ECs we silenced endogenous LPHN2 with human-specific siRNAs and rescued with **silencing resistant (see Fig. S1E) mouse WT or ΔOLF Lphn2 constructs**. **In this regard, we added a further specific statement** in the “Gene silencing in cultured ECs” section of *Materials and Methods* ([page 16, first para](#)).

2. Second, the question of hydrogel stiffness has not been addressed. The authors claim that LPHN2 regulates YAP mechanosignaling, starting from the notion that LPHN2 regulates stress fibers and focal adhesion dynamics, and on the notion that these structures are important for mechanosignaling in general. However, they used hydrogels that are very stiff (in the revised version, even stiffer than in the first), on which YAP is not inhibited. In other words, they do not show that a very soft hydrogel induces YAP inhibition (compared to a stiffer one), and that in those conditions siLPHN2 is sufficient to reactivate YAP. So, the effect of LPHN2 is a basal one, occurring independently of stiffness. Similarly, they did not provide data on LPHN2 regulating YAP localization in confluent monolayers, which would have provided alternative/additional data to gauge this point.

Finally, the fact that LPHN2 is a GPCR acting on cAMP levels and thus likely on PKA indicates it could well regulate the Hippo pathway directly, which has not been addressed experimentally. In absence of data making any specific experimental connection with a mechanical perturbation of ECs, and considering that the effects on YAP might be well due (also? mainly?) to the crosstalk with TJs and/or to a GPCR signaling function, the authors should tone down their claims on mechanosignaling, and focus more in general on Hippo signaling.

As we already discussed in our previous point-by-point rebuttal to Reviewer #3 and first revised version of our manuscript, **we agree with the Reviewer** that the current three-pronged model of the regulation of YAP/TAZ translocation from cytosol to nucleus (Totaro et al., 2018, *Nature Cell Biol.* 20: 888-899; Karaman and Halder, 2018, *Cold Spring Harb. Perspect. Biol.*, 10:a028753; Moya & Halder, 2019, *Nature Rev. Mol. Cell Biol.*, 20: 211–226) must be evaluated. The model key mechanisms that **we carefully addressed and discussed** in our manuscript are:

1. Some [GPCRs](#) can [inhibit](#) YAP/TAZ nuclear translocation *via* [Gαs/AC/cAMP/PKA-dependent inhibition of RHO](#).
2. Integrin-mediated [focal adhesions \(FAs\)](#) and the associated [F-actin](#) stress fibers [promote](#) YAP/TAZ nuclear translocation.
3. ZO-1 containing [tight junctions \(TJs\)](#) [inhibit](#) the nuclear translocation of YAP and TAZ [through Hippo pathway](#)-dependent phosphorylation.

In particular, the revised version of our manuscript includes the following answers to the three above mentioned points:

1. Concerning the possible [involvement of the cAMP/PKA-dependent inhibition of RHO](#) as a pathway by which FLRT2-activated LPHN2 GPCR could inhibit YAP/TAZ nuclear translocation, as shown in [Fig. S1I](#) and discussed on [page 11 \(last paragraph\)](#), we [experimentally addressed](#) and carefully investigated this aspect, and we did [not](#) detect any [increase in RHO activation](#) (GTP-loading) [after LPHN2 silencing in ECs](#). Therefore, we conclude that the GPCR LPHN2 does not inhibit YAP/TAZ nuclear translocation *via* a [Gαs/AC/cAMP/PKA-dependent inhibition of RHO activation](#).

2. Throughout our manuscript we provide formal evidence that [FLRT2-activated LPHN2 inhibits the formation of FAs](#) and the associated [F-actin stress fibers](#). Hence, LPHN2 can inhibit YAP/TAZ nuclear translocation by inhibiting the integrin-driven assembly of FAs. In this regard, [the ability of LPHN2 to directly bind](#) the central PSD-95/Dlg/ZO-1 ([PDZ domain](#)) of the SH3 and multiple ankyrin repeat domains ([SHANK](#)) proteins is likely crucial. Indeed, the binding of the N-terminal Shank/ProSAP N-terminal ([SPN domain](#)) of [SHANK](#) to [Rap1-GTP](#) was found to [suppress talin](#)-mediated integrin activation and FA development (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305). Therefore, LPHN2 may favor the turnover of FAs by funneling Rap1-GTP towards SHANK. We have now formally investigated this hypothesis and found that [in cultured ECs LPHN2 silencing significantly hampers the physical interaction between the small GTPase Rap1 and SHANK2 \(new Fig. S2I\)](#).

It is well established that [integrin-based FAs sense the mechanical features of the microenvironment](#) and regulate the activation of [several mechanosensing pathways, among which YAP/TAZ](#) (Kechagia, Ivaska & Roca-Cusachs. Integrins as biomechanical sensors of the microenvironment. 2019, *Nature Rev. Mol. Cell Biol.*, 20: 457–473). There is as well mounting evidence showing that not only [substrate stiffness, but also ECM ligand density](#) is a [key mechanical feature sensed by integrin-based FAs](#) (Elosegui-Artola et al., *Nat. Cell Biol.*, 2016, 18:540-548; Stanton et al., *ACS Appl. Mater. Interfaces*, 2019, 11, 8849–8857). Remarkably, it has been shown that: i) YAP/TAZ nuclear translocation critically depends on ECM ligand density; ii) at low or high ligand densities, YAP nuclear localization is dominated by ECM ligand density, independently of substrate stiffness; iii) increasing ECM ligand density alone can enhance osteogenic differentiation of human mesenchymal stem cells, regardless of substrate stiffness (Stanton et al., *ACS Appl. Mater. Interfaces*, 2019, 11, 8849–8857). Therefore, together with substrate stiffness, [ECM ligand density](#) plays a [key role in integrin-mediated YAP/TAZ mechanosignaling](#). Therefore, we investigated the impact of LPHN2 signaling on two key mechanical features sensed by integrin-based FAs, namely substrate stiffness and ECM ligand density.

We found that, differently from cancer cells (Dupont et al., *Nature*, 2011, 474:179-183), [endothelial cells \(ECs\)](#) poorly adhere, detach, and [die](#) when plated on very soft ([<1 kPa stiff](#)) [hydrogels](#) necessary to completely inhibit YAP/TAZ activation and nuclear localization in other cell types (0.7 kPa in Dupont et al., *Nature*, 2011, 474:179-183). Since, as shown in the literature, a [complete exclusion of YAP/TAZ from the nucleus is not a prerequisite to study](#) the impact of [integrin-mediated ECM mechanosensing](#) on YAP/TAZ signaling. (*e.g.* see [Oria et al., 2017, Nature, 552:219-224](#)), based on published reports (Birukova et al., 2013, *Microv. Res.*, 87:50–57; Galie et al., 2015, *Lab Chip*, 15:1205-1212; Janmey, Fletcher, & Reinhart-King, Stiffness sensing by cells, 2020, *Physiol. Rev.*, 100:695-724), we sought to perform our experiments on [10 kPa stiffness previously identified as an optimal stiffness for cultured ECs](#) and act on the second key mechanical feature sensed by integrin-based FAs, namely ECM ligand density. As defined in the literature (*e.g.*, see Discher et al., 2009, *Science*, 324:1673-1677; Swift et al., 2013, *Science*, 341, 1240104 - 10.1126/science.1240104; Smith, Cho, and Discher, 2017, *Physiology*, 33:16-25; Guimarães et al., 2020, *Nat. Rev. Mater.* 5:351–370), and as also stated by the Reviewer in the first round of its revision, [5-10 kPa](#) correspond to an [intermediate stiffness](#) (that of a relaxed muscle), while more rigid tissues are typically

characterized by a stiffness higher than 20 kPa (scars) and very rigid tissues display a 30-40 kPa (cartilage) and 100 kPa (pre-calcified bone) stiffness.

In agreement with previous observations (Stanton et al., *ACS Appl. Mater. Interfaces*, 2019, 11, 8849–8857), we found that increasing the density (1, 3, 5 µg/ml) of FN ligand coated on 10 kPa stiff hydrogels proportionally enhances the nuclear translocation of YAP/TAZ in control silenced ECs. Moreover, we found that FLRT2-interacting [LPHN2 significantly inhibits the dose-response translocation of YAP/TAZ in the nucleus of ECs plated on increasing amounts of FN \(Fig. 1K-L and Fig. 2G-H\)](#). Hence, we formally showed that the effect of LPHN2 on YAP/TAZ nuclear translocation also depends on ECM ligand density, a key feature of integrin-dependent mechanosensing.

**3.** Concerning the possible [involvement of TJs](#) in the negative regulation of YAP/TAZ nuclear translocation by FLRT2-activated LPHN2, [we agree](#) with the Reviewer and we further revised our claims and discussion as (s)he requested. In particular, [Rap1](#) was recently formally showed to [promote the formation of TJs](#) (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088). Therefore, as discussed on [pages XX-YY](#), it is [conceivable that LPHN2 activation of Rap1 acts both to inhibit](#) the formation of [FAs](#) and [to promote](#) the assembly of [TJs](#). As discussed in the last part of our further revised manuscript (and depicted in the *schematic* below, corresponding to [Fig. 5E](#)), [the ability of LPHN2 to directly bind](#) the [SHANK](#) proteins is in this regard likely crucial. Indeed, the binding of the N-terminal [SPN domain](#) of [SHANK](#) to [Rap1-GTP](#), in addition to suppress talin-mediated integrin activation and FA development (Lilja et al., 2017, *Nat. Cell Biol.* 19:292–305), [promote](#) the assembly of [TJs](#) (Sasaki et al., 2020, *Cell Rep.* 31:107407 - DOI: 10.1016/j.celrep.2020.02.088). Therefore, LPHN2 may favor the turnover of FAs and the assembly of TJs by funneling Rap1-GTP towards SHANK. As mentioned above (in point 2), we indeed found that [in cultured ECs LPHN2 silencing significantly hampers the physical interaction between the small GTPase Rap1 and SHANK2 \(new Fig. S2I\)](#). Thus, as discussed in the last part of our revised manuscript (and depicted in the *schematic* of Fig. 5E) LPHN2 can inhibit the translocation of YAP/TAZ from cytosol to nucleus by favoring the interaction of active Rap1 with SHANK and the ensuing disassembly of FAs and assembly of TJs.

To summarize, [in agreement with the request of the Reviewer](#) “*the authors should tone down their claims on mechanosignaling, and focus more in general on Hippo signaling*”, we: i) toned down claims on [YAP/TAZ and mechanosignaling](#), which we mentioned only when integrin-mediated cell adhesion to the [ECM](#) was involved downstream of LPHN2; ii) we highlighted the involvement of [Hippo signaling](#) in the control of [YAP/TAZ nuclear translocation](#) when, in agreement with the literature, the formation of [TJs](#) was involved downstream of LPHN2.

August 17, 2021

RE: JCB Manuscript #202006033RR

Prof. Guido Serini  
University of Turin  
Department of Oncology  
Strada Provinciale 142 Km 3.95  
Candiolo 10060  
Italy

Dear Prof. Serini,

Thank you for submitting your revised manuscript entitled "LPHN2 inhibits vascular permeability by differential control of endothelial cell adhesion." Thank you also for your careful attention to reviewer comments and for performing additional experiments. The new data satisfactorily addresses the reviewer points and further strengthens your conclusions. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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