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Lfc subcellular localization and activity is controlled by αv-class integrin

Georgina P. Coló, Andrea Seiwert and Raquel B. Haga

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AUTHORS: Georgina Coló, Andrea Seiwert and Raquel B. Haga
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers consider your observations of interest, but at the same time raise a number of substantial points that prevent me from accepting the paper at this stage. An important point of criticism, raised by reviewers #1 and #2, is the lack of quantification and statistical analysis of your observations. Reviewer #3 has no major concerns except that he/she would like to know whether, like MARK3, MARK2 can directly phosphorylate GEH-H1. Adding to this concern, if MARK2 indeed can phosphorylate GEF-H1 at S151 and the phosphorylation of S151 is critically important in regulating GEF-H1 binding to microtubules, then please explain why the deletion of both MARK2 and 3, produce a more dramatic effect on the binding GEH-H1 to microtubules (Fig. 7). Furthermore, related to this issue, I think it is important to discuss the recent paper by Pasapera et al. in Curr. Biol. (Curr Biol. 32:2704, 2022) on the phosphorylation of myosin II by MARK2. Finally, you will find it perhaps interesting that in a recent study we found that αVβ5 associates with MARK2, GEF-H1 and p115Rho-GEF (Zuidema et al., J. Cell Sci. 135(11):jcs259465). Do you know whether your pKO mouse fibroblasts express αVβ5? I assume they do and could this be a mechanism by which this integrin regulates MARK2 activity.

Because of the various concerns raised by the three reviewers and the additional experimentation required to support your conclusions, I am returning the manuscript to you. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript, which we would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, cells expressing different combinations of integrins (alphaV, beta1 or double alphaV/beta1) have been used to determine how fibronectin signals to activate RhoA. Pulldown experiments identified 9 potential RhoGEFs, with Lfc/ARHGEF2/GEF-H1 emerging as the top candidate based on gene knockout experiments. Further analysis revealed that Lfc phosphorylation induced by integrin engagement activated the protein, with Serine151 being identified as the major site mediating this effect. Activation of integrin signaling leads to S151 phosphorylation, release from microtubules and subsequent RhoA activation. Previous research identified MARK2/MARK3 as mediating S151 phosphorylation, and experiments in this manuscript found that both kinases likely contributed to Lfc phosphorylation and regulation.

Comments for the author

The conclusions are logical, but an overall lack of quantification and statistical analysis reduces the robustness of the conclusions. In particular many conclusions are made based on the localization of Lfc to microtubules, but this has been qualitatively assessed and not quantified, therefore it is not entirely conclusive. It would be important to quantify the observations, and to determine whether there are statistically significant effects, rather than relying on qualitative observations.
Issues to be addressed include the following:

1. It has been described that the three cell types have different actin filament distribution in Figure 1A, but quantitatively how are they different?
Is there more F-actin in some of them? Are the fibers more aligned in some of them? This should be quantified.
2. Figure 1B is a bit confusing, if it's supposed to show that 9 GEFs were pulled down by nucleotide-free RhoA, why are there so many non-GEFs indicated?
What does the grouping to the left actually mean? Other than the patterns of MS results, it's not particularly informative. It would be better to restrict this to the GEFS, and then the grouping to the left might actually be informative.
3. It's not clear how Figure 1C actually is reporting on GEF activity. The graph shows the MS intensity of the GEFs bound to nucleotide-free RhoA, but the intensities would be a function of both the ability of the protein to bind to RhoA and its abundance. Do we know that each of these GEFs is expressed at equal levels in the 3 cell lines? How does their abundance relate to their nucleotide-free RhoA binding?
4. A small suggestion is that the order of the GEFs in Figure 1B should be the same as the order in Figure 1C, it would make it much easier to compare the 2 figures.
5. Why is the important experiment showing that Lfc is the major GEF regulating stress fibres in the supplemental figures? In fact, it's not clear that the depletion of Arhgef2 is actually any different from 3 of the 4 other GEFs, is it actually statistically different from the effects of ArhGEF2 Arhgef11 or Plekhg5?
6. All bar graphs should indicate the individual values from replicate experiments, this level of transparency is essential.
7. The western blot confirming the effect of CRISPR/Cas9 on Lfc protein expression in Supplementary Figure 2 should be mentioned in the text.
8. The effects on F-actin and FAs in Figure 2A should be quantified. It should also be mentioned in the text that the cells were plated on micropatterned substates in the text.
9. Are any of the effects in Figure 2B statistically significant?
10. Co-localization analysis in Figure 3A would be necessary to validate the reported differences in Lfc-MT associations.

11. Quantification of both the Lfc-MT co-localization and cell morphology results in Figure 3B are necessary.
12. Co-staining for MTs is necessary in Supplementary Figure 2B to conclude whether or not there is co-localization of Lfc with MTs.
13. Supplementary Figure 3C needs experimental replicates to make a conclusion about whether there are differences in Lfc activation.
14. The data in Figure 4 could go into supplementary figures, although this is not necessary.
15. The co-localization experiments in Figure 5A should be quantified.
16. An example experiment for Figure 5B should be shown in the figures.
17. Figure 6A should also have staining for MTs, and quantification.
18. Figure 6D should have co-localization quantified.
19. Co-localization in Figure 7A should be quantified. There is no other panel in Figure 7, so there is no need to label it A.

Reviewer 2

Advance summary and potential significance to field

The authors describe a good series of experiments showing that FN attachment of cells through av- or b1-integrins results in different localization of the Rho-GEF LFC. It is shown that LFC is released from MTs in cells attached through av-integrins causing enhanced Rho-binding that may explain the increase in stress fibers. The authors identify MARK2/3 mediated phosphorylation of LFC as the switch involved. A nice study worth publishing pending some additional experiments and textual changes.

Comments for the author

A nice study worth publishing pending some additional experiments and textual changes.

Introduction: the authors present the literature on integrin-regulation of Rho-mediated cytoskeletal organization as if there is only evidence that av-integrins support rhoA. This is a misrepresentation. In different cell systems different findings have pointed to b1- rather than av-mediated activation of RhoA. A more balanced introduction is needed.

Fig 2A: The effect of LFC KO seems similar in all cell types indicating LFC is similarly active in KO-b1 cells.
How do the authors interpret that?
Only one KO line is used. A rescue experiment would be good here. In fact, the authors have expressed WT LFC in these cells (Fig 5). Does that rescue the effect of the KO in this experiment?

Fig 2B. The conclusion in the results section is that “Deletion of Arhgef2 genes decreased RhoA activity in pKO-αv fibroblasts.” But there is no statistics in this figure. Is the effect of LFC KO significant?
Only one KO line is used. A rescue experiment would be good here. In fact, the authors have expressed WT LFC in these cells (Fig 5). Does that rescue the effect of the KO in this experiment?

Fig 2B. Here it seems the KO-av differs from the other two lines whereas in Fig 1 it seems KO-av and KO-avb1 differ from KO-b1. Why is this? Is it absence of b1 or the presence of av that favors Rho activation and Rho-mediated cytoskeletal organization? More extensive description and interpretation of the results is needed here.

Fig 2C. The WB shows more LFC pull down in KO-av and KO-avb1 (even more in the latter) but the quantification shows specifically more in KO-av. Is the quantification the average of multiple WBs? In that case a more representative WB is needed.

Fig S3. The phospho-peptides are even more enriched in KO-avb1 compared to KO-av. Does b1 in fact stimulate such phosphorylation. That would be counter to the message in this paper. More extensive description and interpretation of the results is needed here including why the authors do not consider this supporting a positive role for b1.

Fig 5. Nice experiments clearly identifying phosphorylation of S151 as regulating MT association and RhoA interaction of LFC. Is S151 phosphorylation indeed differentially regulated by av- or b1-mediated FN attachment of cells?

Fig 6: Does staurosporine cause loss of phosphorylation on LFC S151?

Fig 7 and Fig S4: MARK 2 and 3 have been reported to phosphorylate different residues on LFC. Here the KO's cause a rather similar redistribution of LFC to MTs. The S151A and S151D mutants suggested that S151 is the primary site of regulation (Fig 6; S3B). Does LFC-S151D not undergo relocalization to MTs upon MARK2 and/or 3 deletion?

Reviewer 3

Advance summary and potential significance to field

αv-class integrin controls Lfc subcellular localization and activity by Georgina Pamela Coló et al.

Summary: Integrins are known to interact with extracellular matrix components such as fibronectin to trigger actin reorganization and formation of stress fibers and focal adhesions. Stress fiber formation is dependent on the activation of RhoA. This manuscript seeks to elucidate the guanine nucleotide exchange factor that couples integrin activation through fibronectin with RhoA activation and stress fiber formation in fibroblasts.

The authors used a nucleotide free mutant of RhoA to trap active GEFs from fibroblasts lacking av, b1 or avb1 integrins and identify them mass spectroscopy. Using this approach the authors identified LFC/GEF-H1/Arhgef2 in av integrin expressing cells. The authors used CRISPR technology to knockout Arhgef2 in pKO-av, PKO-b1 and pKO-av,b1 fibroblasts and seeded on FN micropatterned substrates. Loss of Arhgef2 resulted in reduced F-actin stress fibers and increased circular peripheral actin. Deletion of Arhgef2 decreased overall RhoA activity in pKO-av fibroblasts. This effect is not seen in pKO-b1 fibroblasts so that the authors conclude that Arhgef2 plays a role in RhoA activation downstream of avb3 integrins. Importantly, pKO-av,b1 fibroblasts exhibited redistribution of Arhgef2 from the microtubules to the cytosol. An avb3 peptidomimetic also stimulated redistribution of Arhgef2 into the cytosol. Conversely a b1 blocking antibody blocked this effect. The authors conclude that av-integrins triggers Arhgef2 release from microtubules. The author then performed phosphoproteomics onArgef2 in each of the knockout cells and identified 5 major phosphorylation sites. Mutation of all of the phosphorylation sites locked Arhgef2 onto the microtubule array. Add-back mutational analysis showed that S151 was critical for regulating Arhgef2 on microtubules (as was previously shown by M. Sandi). The authors go on to show that MARK2/3 are the critical kinases that phosphorylate

Arhgef2 at S151 (as was previously shown by M. Sandi).

Comments for the author

Comments:

This is an interesting and well performed body of experiments that goes a long way to identify Arhgef2 as the critical GEF lying downstream of av-class of integrins in the activation of RhoA and the reorganization of actin. What remains unanswered by this body of work is how av-integrins activate MARK2/3 to phosphorylate Arhgef2 at the regulatory site S151, critically required for its displacement from microtubules to the cytosol where it is active. Anything the authors could do to help answer this question would strengthen the manuscript though it is not required for publication.

First revision

Author response to reviewers' comments

Reviewers' comments:

Editor: An important point of criticism, raised by reviewers #1 and #2, is the lack of quantification and statistical analysis of your observations. Reviewer #3 has no major concerns except that he/she would like to know whether, like MARK3, MARK2 can directly phosphorylate GEH-H1. Adding to this concern, if MARK2 indeed can phosphorylate GEF-H1 at S151 and the phosphorylation of S151 is critically important in regulating GEF-H1 binding to microtubules, then please explain why the deletion of both MARK2 and 3, produce a more dramatic effect on the binding GEH-H1 to microtubules (Fig. 7).

Response:

Quantifications and statistical analyses were included in the new version of the manuscript.

MARK2 has been shown to phosphorylate Lfc in other phospho sites which were reported to also activate Lfc (Yoshimura Y, Terabayashi T, Miki H. Mol Cell Biol. 2010 May;30(9):2206-19). Therefore, even if MARK2 is not affecting directly S151, MARK2 KD could lead to the decrease of phosphorylation of other phospho sites, increasing the effect on the binding of Lfc to microtubules as it was also observed in Fig 4A for the S5A mutant where mutation of 5 serines had a more pronounced effect than S151A alone. This remark was included in the discussion of the paper as the following text: *"MARK2 has been shown to phosphorylate other phospho sites which lead to Lfc activation and translocation to the cytoplasm (Yoshimura and Miki, 2011). Inhibition of the phosphorylation of these sites due to MARK2 depletion in combination to the inhibition of S151 by MARK3 depletion could lead to a more pronounced effect in comparison to depletion of MARK2 or MARK3 alone. The same was observed on Figure 4A, when 5 serines (S5A) were mutated in comparison to S151A alone." (page 11)*

Furthermore, related to this issue, I think it is important to discuss the recent paper by Pasapera et al. in Curr. Biol. (Curr Biol. 32:2704, 2022) on the phosphorylation of myosin II by MARK2. Finally, you will find it perhaps interesting that in a recent study we found that αVβ5 associates with MARK2, GEF-H1 and p115Rho-GEF (Zuidema et al., J Cell Sci. 135(11):jcs259465). Do you know whether your pKO mouse fibroblasts express αVβ5? I assume they do and could this be a mechanism by which this integrin regulates MARK2 activity.

Response:

Although myosin II is a very important protein in actin dynamics, we chose not to discuss it in our system, because according to Schiller et al.'s data (Schiller, H., Hermann, MR., Polleux, J. et al. β1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. Nat Cell Biol 15, 625–636 (2013). https://doi.org/10.1038/ncb2747), quantitative proteomics linked αv-class integrins to Lfc-RhoA pathway coupled to mDia1 but not to myosin, and a5b1 integrins to RhoA-ROCK-myosin II pathway. Since our paper focus on the αv-class pathway, we decided not to include any discussion on the RhoA-ROCK-myosin II pathway to avoid adding too much complexity to the manuscript. However, we mention in the discussion the fact that Pasapera et al. reports the association of MARK2 with FAs which can be relevant to the whole of MARK2 in the αv-class integrin pathway. The following text was added to the manuscript: *"Pasapera et al. has shown that MARK2 can associate with FAs via its membrane-binding domain which suggests that MARKs could be activate by integrins at the FAs." (page 11)*

The paper from Zuidema et al. is a very interesting paper. Our cells express avb5 but since all the experiments were performed on FN, we would assume that avb5 is not playing a role in the mechanism presented on this paper. Indeed, in a normal cell environment, the two integrins (avb3 and avb5) would probably be involved in MARK2 pathway. The following text was added to the manuscript: *"In addition, Zuidema et al. has shown that β5 can be regulated by MARK2 which shows the link of this kinase to the integrin signalling. Indeed, our pKO-av cells express avβ5 integrins. However, since our experiments were performed on FN, avβ5 might not play a role in MARK2 signalling in our system. Therefore, the association of MARK2 signalling with avβ3 integrin*

could happen in the integrin level by a still unknown mechanism or by an unidentified kinase which might operate downstream of av- class integrins and upstream of MARK2/3 in this pathway. A potential candidate is LKB1, which has been shown to positively regulate MARKs (Lizcano et al., 2004, Timm et al., 2008) and to be involved in the translation of integrin signalling into cell polarity response (Chan et al., 2014).” (page 11/12)

Reviewer 1: Advance Summary and Potential Significance to Field: In this manuscript, cells expressing different combinations of integrins (alphaV, beta1 or double alphaV/beta1) have been used to determine how fibronectin signals to activate RhoA. Pulldown experiments identified 9 potential RhoGEFs, with Lfc/ARHGEF2/GEF-H1 emerging as the top candidate based on gene knockout experiments. Further analysis revealed that Lfc phosphorylation induced by integrin engagement activated the protein, with Serine151 being identified as the major site mediating this effect. Activation of integrin signaling leads to S151 phosphorylation, release from microtubules and subsequent RhoA activation. Previous research identified MARK2/MARK3 as mediating S151 phosphorylation, and experiments in this manuscript found that both kinases likely contributed to Lfc phosphorylation and regulation.

Reviewer 1: Comments for the Author: The conclusions are logical, but an overall lack of quantification and statistical analysis reduces the robustness of the conclusions. In particular, many conclusions are made based on the localization of Lfc to microtubules, but this has been qualitatively assessed and not quantified, therefore it is not entirely conclusive. It would be important to quantify the observations, and to determine whether there are statistically significant effects, rather than relying on qualitative observations. Issues to be addressed include the following:

1. It has been described that the three cell types have different actin filament distribution in Figure 1A, but quantitatively how are they different? Is there more F-actin in some of them? Are the fibers more aligned in some of them? This should be quantified.

Response:

Images of F-actin in grey scale were added to Fig 1A to make it easier to visualize the fibres. In addition, quantification of the fluorescence intensity of actin staining and the percentage of cells with ventral stress fibres was done and it is included as Fig 1B. The following text was added to the manuscript: *“Quantification showed that pKO-β1 cells had less fluorescence intensity of actin staining than pKO-av and pKO-av,β1 cells (Figure 1B) and that pKO-β1 and pKO-av,β1 cells exhibit less ventral stress fibres compared to pKO-av cells (Figure 1B).” (page 4)*

2. Figure 1B is a bit confusing, if it’s supposed to show that 9 GEFs were pulled down by nucleotide-free RhoA, why are there so many non-GEFs indicated? What does the grouping to the left actually mean? Other than the patterns of MS results, it’s not particularly informative. It would be better to restrict this to the GEFS, and then the grouping to the left might actually be informative.

Response:

In former Figure 1B, we showed a hierarchical cluster of proteins with the same intensity behaviour in the different samples and conditions. The clusters are automatically generated by the Perseus MS analysis software. The non- GEF proteins are proteins that were also pulled out by GST-tagged RhoA G17A beads, which we used to perform the PD. We agree that this information is not of particular relevance. Therefore, we modified the heat map, adding the whole set of proteins which were detected in the MS and moved it to the Supplementary Fig as Supplementary Fig. 1B. Only the graph showing the LQF relative intensity of the active GEFs that were pulled out by GST-tagged RhoA G17A beads is presented now in the main Figure as Fig 1C.

3. It’s not clear how Figure 1C actually is reporting on GEF activity. The graph shows the MS intensity of the GEFs bound to nucleotide-free RhoA, but the intensities would be a function of both the ability of the protein to bind to RhoA and its abundance. Do we know that each of these GEFs is expressed at equal levels in the 3 cell lines? How does their abundance relate to their nucleotide-free RhoA binding?

Response:

To verify that the MS intensities mirror the activity of RhoA GEFs in the different cell lines we compared the total RhoA GEF levels and found similar expression levels of all GEFs in the three different cell lines. Although active Arhgef5 could be detected in pKO-av cells (however much lower than the other GEFs), total levels of this GEF were not detected in any of our total protein level. The LFQ intensities of the total RhoA GEFs were added as Supplementary Figure 1 C. The following text was added to the manuscript: *"Total proteome revealed equal expression levels of the different GEFs in all three cell lines (Supplementary Figure 1C). Although we could detect low levels of active Arhgef5, enriched via the PD assay, the total protein in our MS assays was not detectable in any of the cell." (page 4)*

4. A small suggestion is that the order of the GEFs in Figure 1B should be the same as the order in Figure 1C, it would make it much easier to compare the 2 figures.
Response:

Following the suggestion in point 2, we have exchanged the heatmap for one showing the hierarchical cluster with the list of all RhoA-G17A binding proteins and moved it to Supplementary Fig 1B.

5. Why is the important experiment showing that Lfc is the major GEF regulating stress fibres in the supplemental figures? In fact, it's not clear that the depletion of Arhgef2 is actually any different from 3 of the 4 other GEFs, is it actually statistically different from the effects of ArhGEF2, Arhgef11 or Plekhg5?

Response:

Supplementary Fig 1B and 1C were moved to Fig 1 (as Fig 1D and Fig 1E). We also added the F-Actin channel in grey to show the differences more clearly. The three GEFs Arhgef2, Arhgef5 and Arhgef11 showed statistical difference to control. However, Arhgef2 shows a more compact and consistent cell pattern, and it had the highest LQF relative intensity in both active and total level GEFs (Fig 1C and Supplementary Fig 1C). In combination with the fact that this GEF was also a hit in a previous publication (Schiller, H., Hermann, MR., Polleux, J. et al. ß1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. Nat Cell Biol 15, 625–636 (2013). https://doi.org/10.1038/ncb2747) using the same cells, we decided to focus on Arhgef2 on this paper, although we cannot discard the role of the other GEFs in this system. The following text was added to the manuscript: *"The experiments revealed that out of the five RhoA GEFs tested, depletion of Lfc/Arhgef2, Arhgef5 and Arhgef11 decreased ventral F-actin stress fibres (Figure 1D and 1E), with Lfc depletion showing the most consistent and highest effect than Arhgef5 and Arhgef 11 depletion (Figure 1E)." (page 5)*

6. All bar graphs should indicate the individual values from replicate experiments, this level of transparency is essential.

Response:

We added the individual values to all possible bar graphs.

7. The western blot confirming the effect of CRISPR/Cas9 on Lfc protein expression in Supplementary Figure 2 should be mentioned in the text.

Response:

The mention of the western blot was already in the text. However, we changed the text in the manuscript to make it clearer as the following: *"To determine how Lfc affects integrin class-specific stress fibre and FA formation, we deleted the Lfc-encoding Arhgef2 genes (Lfc KO,) in pKO-av, pKO-ß1 and pKO-av,ß1 fibroblasts using the Crispr/Cas9 gene editing technology and confirmed the successful KOs via WB (Supplementary Figure 2A)." (page 5)*

8. The effects on F-actin and FAs in Figure 2A should be quantified. It should also be mentioned in the text that the cells were plated on micropatterned substates in the text.

Response:

F-actin and FAs were quantified for all the conditions and were added as Figure 2B and Supplementary Figure 2B. Text was modified to include results of the quantification as following: *"Loss of Lfc expression in pKO-av and pKO av,β1 fibroblasts reduced number of organized stress fibres and length of ventral stress fibres, increased circular actin filaments in the cell periphery, with increase in number of junctions, and resulted in decrease of large FAs (>3 µm) (Figure 2A and 2B, Supplementary Figure 2B). Loss of Lfc in pKO-β1 fibroblasts also led to reduced number of organized stress fibres and length of ventral stress fibres, with loss of central adhesions and a decrease in number of small adhesions (<3 µm) (Figure 2A and 2B, Supplementary Figure 2B)." (page 5)*

The seeding on micropatterns was already mentioned in the text. However, we changed the text in the manuscript to make it clearer as the following: *"The different cell lines were seeded on FN-coated circular micropatterns to analyze F-actin organization (Figure 2A)." (page 5)*

9. Are any of the effects in Figure 2B statistically significant?

Response:

The statistical analysis was added to the graph in Fig 2C (former Fig 2B).

10. Co-localization analysis in Figure 3A would be necessary to validate the reported differences in Lfc-MT associations.
Response:

The co-localization was quantified using the colocalization tool in Zeiss Zen software. The co-localization fraction was determined individually for each cell and the individual thresholds were set accordingly. Several cells were analysed per condition and the results are presented as graphs. The appropriate statistical test was applied.

Quantification was included as Fig 3B.

11. Quantification of both the Lfc-MT co-localization and cell morphology results in Figure 3B are necessary.

Response:

Due to the low number of cells, the co-localization quantification was not possible. To give a better visualization of the staining, the individual channels were added in grey scale as Fig 3C. Since this result is an additional confirmation of the phenotype observed in Fig 3A and 3B, we believe that the missing co-localization analysis would not affect the overall conclusion of the results.
The analysis of the cell morphology was performed, and it was included as Fig 3D.

12. Co-staining for MTs is necessary in Supplementary Figure 2B to conclude whether or not there is co-localization of Lfc with MTs.

Response:

Unfortunately, at the time that the experiment was performed, tubulin staining was not included. Therefore, we performed a line scan analysis to show the variation of GFP-Lfc signal (Supplementary Fig 3A).

13. Supplementary Figure 3C needs experimental replicates to make a conclusion about whether there are differences in Lfc activation.

Response:

Graph showing the quantification and statistical analysis of 3 experimental replicates was included as Supplementary Fig 3C.

14. The data in Figure 4 could go into supplementary figures, although this is not necessary.

Response:

We agree with the suggestion, and we moved Fig 4 to the Supplementary Figures as Supplementary Fig 4. Text was modified as following to accommodate the change: *"The release of Lfc from MTs can occur in cells by depolymerizing MTs, dissociating Lfc from the MT-associated Tctex/DIC complex after LPA/thrombin stimulation and/or direct phosphorylation of Lfc (Krendel et al., 2002, Meiri et al., 2012, Meiri et al., 2014, Fujishiro et al., 2008, von Thun et al., 2013, Patel and Karginov, 2014, Sandí et al., 2017, Yoshimura and Miki, 2011). Since we performed our experiments under serum-free conditions, we can exclude a major involvement of LPA/thrombin-mediated GPCR signalling in the av-class integrin-induced Lfc release from MTs. MT dynamics of the three cell lines were similar after nocodazole washout, MT fractionation and stability assays were performed (Supplementary Figure 4), indicating that the marked Lfc release from MTs in pKO-av is not caused by unstable or depolymerized MTs. Therefore, release of Lfc from MTs in pKO-av could be associated to differential phosphorylation of Lfc in these cells." (page 7)*

15. The co-localization experiments in Figure 5A should be quantified.

Response:

Quantification was done and it was included as Fig 4B (method for quantification is described in item 10).

16. An example experiment for Figure 5B should be shown in the figures.

Response:

We included the WBs as Supplementary Fig. 5C. As the samples containing S151A and S151D were run on separate blots, we highlighted the relevant lanes with dotted rectangles.

17. Figure 6A should also have staining for MTs, and quantification.

Response:

Tubulin staining and quantification of colocalization were added to DMSO and Staurosporine conditions (Fig 5A and 5B). In addition, we added a video that shows the effect of Staurosporine treatment on the microtubules of pKO-av cells as Supplementary Video.

Since we did not have images showing tubulin for the cells treated with NaF and Na3VO4, we moved these results as Supplementary Fig 6A. We believe that the missing tubulin staining for these conditions does not affect the overall observation that Staurosporine treatment affects localization of Lfc in pKO-av cells.

18. Figure 6D should have co-localization quantified.

Response:

Quantification was done and it was included as Fig 5E (method for quantification is described in item 10).

19. Co-localization in Figure 7A should be quantified. There is no other panel in Figure 7, so there is no need to label it A.

Response:

Quantification was done and it was included as Fig 6B (method for quantification is described in item 10).

Reviewer 2: Advance Summary and Potential Significance to Field: The authors describe a good series of experiments showing that FN attachment of cells through av- or b1-integrins results in different localization of the Rho-GEF LFC. It is shown that LFC is released from MTs in cells attached through av-integrins causing enhanced Rho-binding that may explain the increase in stress fibers. The authors identify MARK2/3 mediated phosphorylation of LFC as the switch involved. A nice study worth publishing pending some additional experiments and textual changes.

Reviewer 2: Comments for the Author: A nice study worth publishing pending some additional experiments and textual changes.

Introduction: the authors present the literature on integrin-regulation of Rho-mediated cytoskeletal organization as if there is only evidence that av-integrins support rhoA. This is a misrepresentation. In different cell systems different findings have pointed to b1- rather than av-mediated activation of RhoA. A more balanced introduction is needed.

Response:

The reviewer has a good point and reference to the role of b1 integrins in RhoA activation was included in the introduction as the following: *"Nonetheless, the role of β1 integrins in RhoA activation should not be neglected, as other cell systems showed more pronounced RhoA signalling in presence of b1 integrins compared to avβ3 integrins (Costa et al, 2013; Danen et al. 2002; Vial 2003)." (page 2).*

In addition, another sentence was included to emphasize that av integrin role is more pronounced than b1 integrin in our system, which however does not exclude the involvement of b1 integrins: *"However, how avβ3 integrin activates RhoA in a more pronounced way than a5β1 in fibroblasts and CHO cells is not clear." (page 2).*

Fig 2A: The effect of LFC KO seems similar in all cell types indicating LFC is similarly active in KO-b1 cells. How do the authors interpret that? Only one KO line is used. A rescue experiment would be good here. In fact, the authors have expressed WTLFC in these cells (Fig 5). Does that rescue the effect of the KO in this experiment?

Response:

Although Lfc KO has an effect in all cell lines, the effects on actin organization and FA size is more pronounced in pKO-av cells as evidenced by quantification of the fibres (Fig 2B) and FA size (Supplementary Fig 2B). In addition, depletion of Lfc in pKO-av cells affects activation of RhoA which is not observed when Lfc is depleted in pKO-b1 cells (Fig 2C), indicating that av-class integrin has a greater effect in RhoA signalling in our system. Combining all these results, we decided to focus only on pKO-av cells for the further experiments, but this does not discard that b1 has also a role on actin signalling, as it was already reported by Schiller et al. 2013 (Schiller, H., Hermann, MR., Polleux, J. et al. β1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin- based microenvironments. Nat Cell Biol 15, 625–636 (2013). https://doi.org/10.1038/ncb2747).

Rescue images of all cell lines were added as Supplementary Fig 2C. As expected, re-expression of Lfc rescues the phenotype. The following text was added to the manuscript: *"Re-expression of Lfc in the pKO cells rescued the phenotype of stress fibre formation in all three cell lines (Supplementary Figure 2C)." (page 5)*

Fig 2B: The conclusion in the results section is that "Deletion of Arhgef2 genes decreased RhoA activity in pKO- αv fibroblasts." But there is no statistics in this figure. Is the effect of LFC KO significant? Only one KO line is used. A rescue experiment would be good here. In fact, the authors have expressed WT LFC in these cells (Fig 5). Does that rescue the effect of the KO in this experiment?

Response:

Statistical analysis was added to Fig 2C. Unfortunately rescue experiments to evaluate RhoA activity were not consistent due to the fact that not all the cells re-expressed Lfc and the RhoA activity assay is in general a very variable experiment (at least with our cells), giving us unreliable results. Therefore, rescue experiments for RhoA activity were not conducted any further.

Fig 2B: Here it seems the KO-av differs from the other two lines whereas in Fig 1 it seems KO-av and KO-avb1 differ from KO-b1. Why is this? Is it absence of b1 or the presence of av that favors Rho activation and Rho- mediated cytoskeletal organization? More extensive description and interpretation of the results is needed here.

Response:

Our hypothesis is that av-class integrins favours RhoA activation more than b1 integrins in our system. However, to have a WT phenotype, it is important to have the counter balance of the b1 signalling, otherwise the cells have too many ventral stress fibres and too many adherent FAs. More explanation was added into the discussion as following: *"We find that av-class but not β1 integrins induces high phosphorylation of Lfc and its translocation from MTs to the cytoplasm, followed by Lfc/RhoA activation and stress fibre formation. This does not exclude the role of β1 integrin in activating RhoA in a normal cell setting. However, in our system, activation of RhoA by Lfc was more pronounced in avβ3-only expressing cells compared to b1 integrin expressing cells, and expression of β1 seemed to have a more counter balance effect to avβ3 signalling on RhoA activation as evidenced by pKO-av,β1 phenotype. pKO-av,β1 cells, which show similarity with the WT phenotype, showed almost as much activation of Lfc as pKO-av cells, however similar RhoA activation as pKO-β1 cells. This indicates that although avβ3 stimulates the activation of Lfc, if a5β1 is present, levels of RhoA activation will remain as WT levels." (page 10)*

Fig 2C: The WB shows more LFC pull down in KO-av and KO-avb1 (even more in the latter) but the quantification shows specifically more in KO-av. Is the quantification the average of multiple WBs? In that case a more representative WB is needed.

Response:

A more representative WB was added in Figure 2D (former Fig 2C).

Fig S3: The phospho-peptides are even more enriched in KO-avb1 compared to KO-av. Does b1 in fact stimulate such phosphorylation. That would be counter to the message in this paper. More extensive description and interpretation of the results is needed here including why the authors do not consider this supporting a positive role for b1.

Response:

Our hypothesis is that the phospho-peptides are even more enriched not because of b1 directly, but because b1 activates the inhibitory phospho-sites S885 and S959 to counter balance the signalling and therefore, since the cells sense inhibition of signalling, there is a feedback loop to increase activation of the phospho-sites by avb3, increasing therefore the activation of these sites. A partial explanation was already in the text, and an additional text was added to make this point clearer as follows: *"Interestingly, not only do pKO-av,β1 cells show high activation of the same phospho-sites as pKO-av cells but also of S885 and S959 (Supplementary Fig 5A), which have been linked to Lfc inhibition (Yamahashi et al., 2011). This might hint to a balanced Lfc regulation in these cells, where both avβ3 and a5β1 integrins are expressed, leading to an even higher activation of the phospho-sites observed for the pKO-av cells to balance the activation of the inhibitory sites S885 and S959 by a5β1 integrins. Since activation of Lfc/RhoA and formation of thick stress fibres connected to large FAs were less pronounced in pKO-av,β1 cells compared to pKO-av cells, this further supports our hypothesis that avβ3 and a5β1 integrins counter balance each other in regulating the Lfc/RhoA signalling in WT mouse fibroblasts." (page 10/11)*

Fig 5: Nice experiments clearly identifying phosphorylation of S151 as regulating MT association and RhoA interaction of LFC. Is S151 phosphorylation indeed differentially regulated by av- or b1-

mediated FN attachment of cells?

Response:

The heat map on Supplementary Figure 5A shows that activation of S151 is higher in cells expressing av-class integrin than in cells expressing b1-integrin only.

Fig 6: Does staurosporine cause loss of phosphorylation on LFC S151?

Response:

Treatment of pKO-av cells with Staurosporine leads to decrease in activation of many phospho-sites, including S151. The heat map of the unbiased phospho-enrichment proteomic analysis after Staurosporine-treated pKO-av cells was added as Supplementary Figure 6B and the following text was added to the manuscript: *"Treatment of pKO-av with Staurosporine decreased overall Lfc phosphorylation, including phosphorylation of S151 as observed by unbiased phospho-enrichment proteomic analysis (Supplementary Figure 6B and 6C)." (page 9)*

Fig 7 and Fig S4: MARK 2 and 3 have been reported to phosphorylate different residues on LFC. Here the KO's cause a rather similar redistribution of LFC to MTs. The S151A and S151D mutants suggested that S151 is the primary site of regulation (Fig 6; S3B). Does LFC-S151D not undergo relocalization to MTs upon MARK2 and/or 3 deletion?

Response:

We did not perform this experiment because the cells already don't look very healthy after MARK2/3 depletion, and the transfection of an additional construct would lead to more cell stress. In addition, this experiment would show that other sites, apart from S151, might contribute for the phenotype observed in Fig 6A (former Fig 7), since Lfc- S151D is mainly in cytoplasm as observed in Fig 5D and Supplementary Fig 5B.

Reviewer 3: Advance Summary and Potential Significance to Field: αv-class integrin controls Lfc subcellular localization and activity by Georgina Pamela Coló et al.

Summary: Integrins are known to interact with extracellular matrix components such as fibronectin to trigger actin reorganization and formation of stress fibers and focal adhesions. Stress fiber formation is dependent on the activation of RhoA. This manuscript seeks to elucidate the guanine nucleotide exchange factor that couples integrin activation through fibronectin with RhoA activation and stress fiber formation in fibroblasts. The authors used a nucleotide free mutant of RhoA to trap active GEFs from fibroblasts lacking av, b1 or avb1 integrins and identify them mass spectroscopy. Using this approach the authors identified LFC/GEF-H1/Arhgef2 in av integrin expressing cells. The authors used CRISPR technology to knockout Arhgef2 in pKO-av, PKO-b1 and pKO-av,b1 fibroblasts and seeded on FN micropatterned substrates. Loss of Arhgef2 resulted in reduced F-actin stress fibers and increased circular peripheral actin. Deletion of Arhgef2 decreased overall RhoA activity in pKO-av fibroblasts. This effect is not seen in pKO-b1 fibroblasts so that the authors conclude that Arhgef2 plays a role in RhoA activation downstream of avb3 integrins. Importantly, pKO-av,b1 fibroblasts exhibited redistribution of Arhgef2 from the microtubules to the cytosol. An avb3 peptidomimetic also stimulated redistribution of Arhgef2 into the cytosol.

Conversely a b1 blocking antibody blocked this effect. The authors conclude that av-integrins triggers Arhgef2 release from microtubules. The author then performed phosphoproteomics onArgef2 in each of the knockout cells and identified 5 major phosphorylation sites. Mutation of all of the phosphorylation sites locked Arhgef2 onto the microtubule array. Add-back mutational analysis showed that S151 was critical for regulating Arhgef2 on microtubules (as was previously shown by M. Sandi). The authors go on to show that MARK2/3 are the critical kinases that phosphorylate Arhgef2 at S151 (as was previously shown by M. Sandi).

Reviewer 3: Comments for the Author: This is an interesting and well performed body of experiments that goes a long way to identify Arhgef2 as the critical GEF lying downstream of av-class of integrins in the activation of RhoA and the reorganization of actin. What remains

unanswered by this body of work is how av-integrins activate MARK2/3 to phosphorylate Arhgef2 at the regulatory site S151, critically required for its displacement from microtubules to the cytosol where it is active. Anything the authors could do to help answer this question would strengthen the manuscript though it is not required for publication.

Response:

At this point, we don't have more experiments to support this question. As shown by Zuidema et al. (Zuidema et al., J. Cell Sci. 135(11):jcs259465), integrins (in this case b5) can interact with MARK2 and be regulated by it. Therefore, we expect that interaction of av-class integrins with MARKs could happen directly or indirectly, but at this point we don't have enough data to suggest a mechanism.

In addition:

- Reference to Figures was modified throughout the text to follow the changes.
- Legends were modified to follow the changes in the Figures.
- Description of the methods that were used to generate the new data was added to the Materials and Methods section.
- All changes are highlighted in yellow in the manuscript.

Second decision letter

MS ID#: JOCES/2022/260740

MS TITLE: αv-class integrin controls Lfc subcellular localization and activity

AUTHORS: Georgina Coló, Andrea Seiwert and Raquel B. Haga
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers #2 and #3 find that you have satisfactorily addressed their comments and recommend publication. However, reviewer #1 still has a minor issue that will need to be adressed before submitting your final manuscript. I hope that you will be able to carry this out .

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, cells expressing different combinations of integrins (alphaV, beta1 or double alphaV/beta1) have been used to determine how fibronectin signals to activate RhoA. Pulldown experiments identified 9 potential RhoGEFs, with Lfc/ARHGEF2/GEF-H1 emerging as the top candidate based on gene knockout experiments. Further analysis revealed that Lfc phosphorylation

induced by integrin engagement activated the protein, with Serine151 being identified as the major site mediating this effect. Activation of integrin signaling leads to S151 phosphorylation, release from microtubules and subsequent RhoA activation. Previous research identified MARK2/MARK3 as mediating S151 phosphorylation, and experiments in this manuscript found that both kinases likely contributed to Lfc phosphorylation and regulation.

Comments for the author

The manuscript has been considerably improved during the revisions, the major issue of lack of quantification has been satisfactorily addressed. There is one remaining issue: Figure 1B shows that there are statistically significant effects of C3 treatment of each cell line, but the text on MS page 9 claims that there are differences between cell lines. This hasn't been subjected to statistical analysis, and should be in order to make these conclusions.

Reviewer 2

Advance summary and potential significance to field

As in my first review

Comments for the author

The authors have added additional data to support several conclusions. The intro is more balanced, now referring also to work pointing to b1 integrin-mediated rho activation. The rescue experiments for the single KO data strengthen the paper. It is a pity that this cannot be done for the data on Rho activation (too challenging as re-expression is not in all cells) but given the data on actin organisation this is acceptable. The work around the phosphorus-proteomics heat map is not further extended - it would have been good to see stronger follow-up data on S151 as a key site of regulation, as requested by the reviewer. Nevertheless, the paper can be published.

Reviewer 3

Advance summary and potential significance to field

Integrins are known to interact with extracellular matrix components such as fibronectin to trigger actin reorganization and formation of stress fibers and focal adhesions. Stress fiber formation is dependent on the activation of RhoA.
This manuscript seeks to elucidate the guanine nucleotide exchange factor that couples integrin activation through fibronectin with RhoA activation and stress fiber formation in fibroblasts. The authors used a nucleotide free mutant of RhoA to trap active GEFs from fibroblasts lacking av, b1 or avb1 integrins and identify them mass spectroscopy. Using this approach the authors identified LFC/GEF-H1/Arhgef2 in av integrin expressing cells. The authors used CRISPR technology to knockout Arhgef2 in pKO-av, PKO-b1 and pKO-av,b1 fibroblasts and seeded on FN micropatterned substrates. Loss of Arhgef2 resulted in reduced F-actin stress fibers and increased circular peripheral actin. Deletion of Arhgef2 decreased overall RhoA activity in pKO-av fibroblasts. This effect is not seen in pKO-b1 fibroblasts so that the authors conclude that Arhgef2 plays a role in RhoA activation downstream of avb3 integrins. Importantly, pKO-av,b1 fibroblasts exhibited redistribution of Arhgef2 from the microtubules to the cytosol. An avb3 peptidomimetic also stimulated redistribution of Arhgef2 into the cytosol.
Conversely a b1 blocking antibody blocked this effect. The authors conclude that av-integrins triggers Arhgef2 release from microtubules. The author then performed phosphoproteomics onArgef2 in each of the knockout cells and identified 5 major phosphorylation sites. Mutation of all of the phosphorylation sites locked Arhgef2 onto the microtubule array. Add-back mutational analysis showed that S151 was critical for regulating Arhgef2 on microtubules (as was previously shown by M.
Sandi). The authors go on to show that MARK2/3 are the critical kinases that phosphorylate Arhgef2 at S151 (as was previously shown by M. Sandi).

Comments for the author

This manuscript is now acceptable for publication

Second revision

Author response to reviewers' comments

Reviewers' comments:

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Response:

Thanks for the observation. In agreement with the reviewer suggestion, statistical analyses between pKO-av and pKO-b1, pKO-av and pKO-av,b1, and pKO-b1 and pKO-av,b1, were included in Fig 1B. Two small changes were made to the Fig 1 Legend to add this new information, The changes are highlighted in yellow.

Reviewer 2 Advance Summary and Potential Significance to Field:
As in my first review

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The authors have added additional data to support several conclusions. The intro is more balanced, now referring also to work pointing to b1 integrin-mediated rho activation. The rescue experiments for the single KO data strengthen the paper. It is a pity that this cannot be done for the data on Rho activation (too challenging as re- expression is not in all cells) but given the data on actin organisation this is acceptable. The work around the phosphorus-proteomics heat map is not further extended - it would have been good to see stronger follow-up data on S151 as a key site of regulation, as requested by the reviewer. Nevertheless, the paper can be published.

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Reviewer 3 Comments for the Author:

This manuscript is now acceptable for publication

Third decision letter

MS ID#: JOCES/2022/260740

MS TITLE: αv-class integrin controls Lfc subcellular localization and activity

AUTHORS: Georgina Coló, Andrea Seiwert and Raquel B. Haga
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.