An optogenetic model reveals cell shape regulation through FAK and fascin

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AUTHORS: Jean A Castillo-Badillo and N Gautam
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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 gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

This study utilizes a clever optogenetic tool, based on a light-inducible dimerization component that includes a GEF for RhoA, to activate RhoA in the center of a mononucleated macrophage and induce contraction thereby causing changes in cell shape that are associated with cleavage furrow formation. As the nucleus gets pushed to the side, the non-nucleated portion of the cell lacks the mechanical support provided by the nucleus and collapses. This manipulation allows for the study of mechanisms involved in cell shape maintenance during furrow formation of nascent daughter cells, which lack the mechanical support provided by a nucleus. The authors use the model to study how two effectors of RhoA, focal adhesion kinase (FAK)
and the actin-bundling protein, fascin, contribute to maintenance of cell shape in the absence of nuclear support. Through overexpression approaches or treatment with pharmacological inhibitors, the authors provide evidence that FAK is involved in regulating adhesion area, while fascin is involved in regulating cell height. The author's interpretations of their observations are reasonable and based on known functions of FAK and fascin. However, the findings are largely phenomenological, so the main impact lies in the application of the model to address long-standing questions regarding mechanisms that control cell shape. It is therefore important that the study's rigor be improved by addressing several weaknesses.

Comments for the author

Major weaknesses:
1. How was it determined that 30 min treatment with 10um FAK14i was the optimal treatment condition for this inhibitor? The authors should provide western blots or other confirmation that FAK14i effectively inhibits FAK phosphorylation in their cells under these conditions.

2. It is possible that FAK14i has some non-specific or off-target effects. To robustly support their conclusions, the authors should include experiments using an independent approach (e.g., siRNA) to inhibit or suppress FAK.

3. Similarly, it is not clear why 30 min treatment with 40um G2 was chosen to inhibit fascin, or whether this small molecule inhibitor might have non-specific or off-target effects. Here, too, support for the conclusions would be strengthened by inclusion of siRNA experiments to suppress fascin.

Minor issues:
4. The order of panels D and E in Figure 2 should be switched so that they match the discussion order in the text.

5. It is not immediately intuitive why a more spherical cell would be more rigid than a cell that is adhered to a rigid support (lines 253-255, and 392-394). What is the support for this statement?

6. It is not clear what the authors intend to depict in the illustration shown in Figure 7.

Reviewer 2

Advance summary and potential significance to field

In the manuscript of Castillo-Badillo and Gautam, the authors use an innovative optogenetic approach to induce cleavage furrow formation in mononucleated macrophages spatiotemporally controlled by blue light and analyze the resulting cell shape changes in response to FAK and fascin overexpression or inhibition. In particular, the authors were interested in the non-nucleated side upon cleavage furrow formation where the nucleus cannot provide mechanical support. They found that FAK overexpression maintains adhesion area whereas fascin overexpression maintains cell height during cell shape changes induced by furrow formation. Inhibition of FAK or fascin prevented furrow formation and associated cell shape changes highlighting the role of the two proteins in cell shape regulation. The analysis of the cell height upon optogenetic global RhoA activation in NIH3T3

cells revealed an increase in cell height in cells overexpressing fascin whereas the cell height of non-modified cells remained constant. This experiment supports the role of fascin in cell height regulation as it was performed in different cells and in the absence of furrow formation. In summary, the authors provide the community with new and important insight how FAK and fascin are involved in regulating cell shape.

Comments for the author

The study is highly interesting and well performed, the manuscript is nicely written and I certainly can support publication of this manuscript after the following minor issues have been addressed:

- In line 153-155, the authors state that the nucleated side maintains a similar morphology with comparable height and adhesion area to the pre-photoactivated cell. However, this statement is not supported by a quantitative analysis of the images. The authors should include in Figure 1 a quantitative analysis similar as in Figure 2D and E. Please note that the upper part of the XZ projection in Figure 1C seems to be missing / not imaged.
- In the different figures, a z-stack was acquired at the beginning and end of each experiment. However, it is not stated at which time exactly the z-stack at the end of the experiment was imaged. Please include this information in all related figures.
- In Figure 2, 3 and 6 the quantitative analysis of the cell height and area is only performed from a part of the acquired images (e.g. In Figure 2A the image is representative of 42 of 46 cells but the quantitative analysis in 2D and 2E was only done with 11 cells). What is the motivation behind this selection of the cells? I would highly recommend to include all images in the quantitative analysis although a statistically significant effect could already be observed with less cells.
- In Movie S3 and S6 the focus of the experiment is the height of the cell. However, this information cannot be obtained from the movies as they are only showing one focal plane over time. The authors should include the height projections (XZ, YZ) in the movies similar to Figure 1B, C. Moreover the authors should include a scale bar in all movies or refer to the corresponding figure in the manuscript for the scale bar.
- Although the utilized optogenetic tool was published already previously, I think a scheme of the optogenetic tool at the beginning of the manuscript would facilitate understanding of the manuscript by readers outside the field.
- I would suggest not to use the term “mathematical analysis” in the abstract as it suggests a complex mathematical analysis or model. As rather a simple approach to analyze cell height and area was used, I think “quantitative analysis” would be more appropriate.
- For n.s. = no significant difference, please include the exact P value similar to the statistically significant changes.
- In Figure 2A at 6:30 the yellow illumination box seems to be missing.
- In Figure 4B the green color represents H2B-Venus and not Venus-KRasCT as stated in the image.
- In Figure 7 it is almost not visible that the actin filaments are dotted (as indicated in the legend).
- In their optogenetic experiments, the authors used a 514/515 nm laser for Venus imaging. Can imaging with this laser also activate (low amounts of) RhoA?
- To facilitate reproduction of the shown experiments by other researchers, I would highly recommend to substantially extend the material and methods part as follows: 1) Please include more information about the used and cloned plasmids. What fascin fragment was exactly used (which amino acids)? What it the amino acid sequence of the spacers? Which plasmids/promoters were used for LARG-mTurq-SspB, Venus-KRasCT and iLID-Caax? I would suggest to give at least the amino acid sequences of the proteins encoded by plasmids cloned in this study in the supplement. 2) Information about the amount of transfected plasmid is missing. Which ratios between the different plasmids were used in which experiment? What was the total plasmid amount? What to the authors mean with cDNA? Based on the methods I was thinking that the authors rather transfected plasmid DNA? Please clarify. 3) The cells were imaged after 4-10 hours after transfection. This is very early and I would assume very low expression levels. What was the motivation behind this short incubation time? Is this incubation time important for the outcome of the experiments? Does longer expression

result in spontaneous activation of RhoA due to the higher protein level? 4) What blue light intensity was used with the DMD?

Reviewer 3

Advance summary and potential significance to field

The authors aim at exploring the mechanisms regulating cell shape. To do so, they use an optogenetic tool that activates RhoA upon blue light stimulation and activate cleavage furrow formation in mononucleated macrophages. This allows them to obtain 2 “half cells”, one containing the nucleus and one that does not. They then go on to study the role of FAK and Fascin in regulating cell shape, in particular using cell height and cell/substrate adhesion as a proxy.

Overall, this is an interesting, clear and well written manuscript, using an elegant approach to investigate cell shape with an internal control. However, I do feel that some claims are not sustained by the data, but that in most cases simple quantifications and controls could remedy this. Thus, it is my recommendation that this paper be accepted for publication pending appropriate revisions.

Comments for the author

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Major:
1) I am a little confused about Figure 2. Are the quantifications in panels D-E performed in FAK overexpressed optogenetically activated cells? Can the authors compare to WT optogenetically activated cells to drive the point that in FAK overexpressed cells the adhesion patterns of the two parts of the cell is driven by FAK? Similarly, how does the height of the part that does contain the nucleus compare with or without FAK overexpression? It seems like the while the height of the non-nucleated half is still smaller than its nucleated counterpart, it is also larger than in controls from figure 1 (which should also be quantified), which suggest that FAK/or FAK mediated adhesion also plays a part in mediating cell height. Could the authors discuss?
The authors also claim that there is a higher FAK expression in regions distal to the furrow. This is not obvious from the figure, can this be quantified?

2) I have the exact same comments on Figure 3. I think the 2 parameters (height, adhesion surface) should be compared to control cells. Otherwise, the authors can only conclude about the role of fascin (or FAK in the case of Figure 2) in the differential shapes between nucleated and non nucleated, but cannot make general claims about the role of fascin or FAK on cell shape.
The authors should also quantify their claim that fascin expression is higher in the non nucleated part (using a Z-plan that does not contain the nucleus). In particular, in this case, it is important to look at fascin underlying the actin cortex, since it is doubtful fascin in the cytoplasm plays any part.

3) Figure 4C, it seems like RhoA activation in Fascin overexpressed cells leads to an elongation of the cell, possibly by spreading on the substrate. Is that true? Could the authors quantify this?

4) Line 371: the authors claim that the persistence of cell shape changes are due to higher level of FAK maintaining a greater adhesion. This is not clear from the data. The authors should test this by repeating the experiment on micropatterns of different adhesion (either modulating the strength of the adhesion via coating eg https://www.nature.com/articles/s41467-020-20563-9, or maybe the size of the adhesion pattern).

5) Induction of changes in local contractility leading to a furrow-like structure in mononucleated cells cannot be directly compared to the processes involved in controlling cell shape during mitosis. Despite the absence of nucleus and a characteristic organisation of the actin network, other cytoskeletal components important for maintaining cell shape drastically reorganise during cell division, impact cell surface mechanics and affect cell shape. While the findings of the study can be used to infer the potential mechanisms controlling cell shape during mitosis, the authors should not use the direct comparison. The phenotype observed in this study might be more relevant for the study of amoebal migration then cell division. Therefore, the authors should tone down their claim that this model allows a direct comparison with mitosis.
In a side note, the data in Movie S1, Figure 1 and Movie S5 and Figure 5 suggests that the system presented could be an interesting model of migration. Therefore, quantifying data related to migration (such as speed and direction) upon RhoA photoactivation might offer interesting insights about mechanisms for migration. This is beyond the scope of this paper but could be interesting in the future.

6) The authors should change the bar graphs to dot plots, to be more transparent about the number of points, especially since the authors use very small n numbers and SEM instead of SD. In the few quantified experiments, the n number is pretty low, and the paper would benefit from adding some data points if possible (and in particular, the experiments presented in figure 1 should be quantified, and SD should be used over SEM for n=45).

Minor:
In the introduction, the authors suggest that the existing experimental methods to study cell shape changes are limited to growing cells on different surfaces ignoring a variety of confinement methods. Manipulation of cell shape through more biochemical approaches of depletion, overexpression or use of inhibitors is also ignored. Since the optogenetic approach induces cell shape through activation of RhoA and thus signalling, more traditional biochemical approaches are also relevant. The authors should amend the text to not ignore this body of literature.
Line 181: it should be clear that FAK is overexpressed in these cells. Could the authors quantify the amount of overexpression by western blot?
Line 371: the authors say that the role of fascin in maintaining cell height is counterintuitive. I would argue that published in vitro and in silico data suggest differently (Chugh at al., 2017; Ennomani et al., 2016; Ding et al., 2017). Fascin is an actin crosslinker: more crosslinking will lead to a higher tension and thus a rounding of the cells.
Furthermore, there are multiple different actin bundling proteins present in the cells. The selection of fascin over more commonly used alpha-actinin 4 is somewhat surprising and I believe authors should explain why they chose this particular actin bundler. Additional discussion and data with other actin bundlers might also confirm whether the observed mechanisms is due to the bundling activity of fascin. The authors might also be interested in findings by Logue et al., 2015 where actin bundler Eps8 is shown to affect migration. Since the model is applied to a cell division, Dix et al. paper could be discussed in relation with focal adhesion part of the model.

The discussion should be toned down, it should be clear where the authors are hypothesising. For ex line 439-442, the authors have not shown that FAK is more recruited distally, and they have not shown that the bottom of the cell is more stable. If they want to claim this, then they should show that it is more stable, for example by showing and quantifying kymographs of the plasma membrane in that bottom plane.
Similarly, line 461-462, it is not clear that fascin is present in higher quantities on the side without a nucleus, as it has not been properly quantified.
The authors just justify the use of a t test (by at least saying their data follows a normal distribution). Furthermore, if they have n=42 cells (as presented in 2A for example), they should analyse 42 cells and not 11 (as presented in 2D for example) or justify why they cannnot.
A few typos:
line 81: Repeated punctuation line 113: Missing reference for regulation of FAK and fascin by RhoA.
line 142: mono-nucleated should be mononucleated

First revision

Author response to reviewers' comments

We thank the reviewers for valuable comments that have helped improve the manuscript. In response to a comment from Reviewer 3 we have performed TIRF experiments to show that there is a dramatic increase in adhesion in cells expressing FAK.

Reviewer 1 Comments for the Author:

Major weaknesses:

1. How was it determined that 30 min treatment with 10um FAK14i was the optimal treatment condition for this inhibitor? The authors should provide western blots or other confirmation that FAK14i effectively inhibits FAK phosphorylation in their cells under these conditions.

Existing literature characterizing FAK14i has shown that it acts in a dose- and time- dependent manner, with FAK inhibition detected at 10 μM (Golubovskaya et al., 2008). Because the need to image live electroporated RAW cells within hours after transfection, incubation times of several hours are not feasible. Additionally, for the purposes of our study it was not necessary to induce complete inhibition of FAK, since even partial inhibition is sufficient to demonstrate the role of FAK on the cell's ability to maintain shape changes.

2. It is possible that FAK14i has some non-specific or off-target effects. To robustly support their conclusions, the authors should include experiments using an independent approach (e.g., siRNA) to inhibit or suppress FAK.

Extensive characterization of this inhibitor has demonstrated via Western blotting that FAK14i inhibits FAK phosphorylation at site Y397 with high specificity, and the observed phenotypic effects, such as changes in cell adhesion and detachment, were related to FAK inhibition (Golubovskaya et al., 2008). Inhibition with siRNA is initiated as soon as the inhibitory RNA is expressed or introduced. This inhibition is thus over a much longer period compared to the acute inhibition with a chemical inhibitor. It can therefore have potential secondary effects that may cloud interpretation.

3. Similarly, it is not clear why 30 min treatment with 40um G2 was chosen to inhibit fascin, or whether this small molecule inhibitor might have non-specific or off-target effects. Here, too, support for the conclusions would be strengthened by inclusion of siRNA experiments to suppress fascin.

This inhibitor, G2, has also been extensively characterized and been shown to have a Kd of 5-20 μM (Huang et al., 2015). Based on this evidence we chose 40 μM to ensure *effective fascin inhibition. As mentioned above we think acute inhibition with well characterized inhibitors is advantageous over siRNA.*

Minor issues:

4. The order of panels D and E in Figure 2 should be switched so that they match the discussion order in the text.

We changed the order of the panels D and E in Figure 2.

5. It is not immediately intuitive why a more spherical cell would be more rigid than a cell that is adhered to a rigid support (lines 253-255, and 392-394). What is the support for this statement?

We have rewritten these parts to make it more clear. As can be seen in control cells in Figure 1, macrophages are naturally spherical in their basal state, especially when compared to naturally flat cells like fibroblasts. Overexpression of fascin, which is known to make the cytoskeleton more rigid, would make the cell less likely to deviate from its native spherical shape, allowing it to maintain its height while undergoing shape changes.

Reviewer 2 Comments for the Author:

The study is highly interesting and well performed, the manuscript is nicely written and I certainly can support publication of this manuscript after the following minor issues have been addressed:

1. In line 153-155, the authors state that the nucleated side maintains a similar morphology with comparable height and adhesion area to the pre-photoactivated cell. However, this statement is not supported by a quantitative analysis of the images. The authors should include in Figure 1 a quantitative analysis similar as in Figure 2D and E. Please note that the upper part of the XZ projection in Figure 1C seems to be missing / not imaged.

We addressed this suggestion by quantifying the relative change in height and adhesion area in response to photoactivation. The resulting graph (Figure 1E) confirms that no significant change in height or adhesion area occurs in response to photoactivation in cells with wild type levels of FAK and fascin.
We have replaced Fig 1C with a corrected image.

2. In the different figures, a z-stack was acquired at the beginning and end of each experiment. However, it is not stated at which time exactly the z-stack at the end of the experiment was imaged. Please include this information in all related figures.
We added the start point time (00:00) for the Z-stack images before photoactivation and the ending point time (approximately 8 min) for the Z-stack images after photoactivation to Figures 1C-D, 2B-C, and 3B-C.

3. In Figure 2, 3 and 6 the quantitative analysis of the cell height and area is only performed from a part of the acquired images (e.g. In Figure 2A the image is representative of 42 of 46 cells but the quantitative analysis in 2D and 2E was only done with 11 cells). What is the motivation behind this selection of the cells? I would highly recommend to include all images in the quantitative analysis although a statistically significant effect could already be observed with less cells.

We appreciate the reviewer's suggestion; however, the 46 cells referred to in Figure 2A were assayed in a single confocal plane. Due to the time-consuming nature of single cell assays and challenging experimental methodology associated with optogenetics, z- stacks before and after photoactivation were only acquired for 11 of those 46 cells. These 11 cells were then quantified in Figures 2D-E.

4. In Movie S3 and S6 the focus of the experiment is the height of the cell. However, this information cannot be obtained from the movies as they are only showing one focal plane over time. The authors should include the height projections (XZ, YZ) in the movies similar to Figure 1B, C. Moreover, the authors should include a scale bar in all movies or refer to the corresponding figure in the manuscript for the scale bar.

Movies S3, S6 and all other movies were captured in a single confocal plane that corresponds to the middle plane of the cell. We found that repeated exposure to 514 nm laser light, which is necessary to acquire a time series z-stack, causes global photoactivation of our optogenetic tool. This makes it impossible to polarize RhoA signaling and prevents furrow formation in any condition. For that reason, we performed the experiment in a single confocal plane and only acquired z-stack images at the beginning and end points.
With regard to scale bars we have referred to the corresponding figure.

5. Although the utilized optogenetic tool was published already previously, I think a scheme of the optogenetic tool at the beginning of the manuscript would facilitate understanding of the manuscript by readers outside the field.

We addressed this suggestion and added scheme of the optogenetic tool in Figure 1A.

6. I would suggest not to use the term "mathematical analysis" in the abstract as it suggests a

complex mathematical analysis or model. As rather a simple approach to analyze cell height and area was used, I think "quantitative analysis" would be more appropriate.

We agree with the reviewer and we have replaced "mathematical analysis" with "quantitative analysis", which we agree is more appropriate.

7. For n.s. = no significant difference, please include the exact P value similar to the statistically significant changes.

We added the P values in figure legends for all the plots, including in cases with no significant difference.

8. In Figure 2A at 6:30 the yellow illumination box seems to be missing.

We corrected Figure 2A to include the light box.

9. In Figure 4B the green color represents H2B-Venus and not Venus-KRasCT as stated in the image.

We corrected the label in Figure 4B to H2B-Venus.

10. In Figure 7 it is almost not visible that the actin filaments are dotted (as indicated in the legend).

We changed the figure legend to indicate that the line is solid.

11. In their optogenetic experiments, the authors used a 514/515 nm laser for Venus imaging. Can imaging with this laser also activate (low amounts of) RhoA?

We found that overexposure to the 514/515 nm laser can indeed induce global photoactivation of our optogenetic construct. This global photoactivation can make it difficult to localize RhoA signaling to a specific area of the cell and is one of the reasons why it is challenging to acquire z-stacks in time lapse. We therefore performed the experiments with short exposure times and low power for the 514 laser, allowing for localized photoactivation of RhoA.

12. To facilitate reproduction of the shown experiments by other researchers, I would highly recommend to substantially extend the material and methods part as follows: 1) Please include more information about the used and cloned plasmids. What fascin fragment was exactly used (which amino acids)? What it the amino acid sequence of the spacers? Which plasmids/promoters were used for LARG-mTurq-SspB, Venus- KRasCT and iLID-Caax? I would suggest to give at least the amino acid sequences of the proteins encoded by plasmids cloned in this study in the supplement. 2) Information about the amount of transfected plasmid is missing. Which ratios between the different plasmids were used in which experiment? What was the total plasmid amount? What to the authors mean with cDNA? Based on the methods I was thinking that the authors rather transfected plasmid DNA? Please clarify. 3) The cells were imaged after 4-10 hours after transfection. This is very early and I would assume very low expression levels. What was the motivation behind this short incubation time? Is this incubation time important for the outcome of the experiments? Does longer expression result in spontaneous activation of RhoA due to the higher protein level? 4) What blue light intensity was used with the DMD?

1) We included the amino acid sequence for Venus-Fascin and mCherry-Fascin in the Supplemental Materials. For LARG-mTurq-SspB, Venus-KRasCT and iLID-CaaX we included the Addgene number in the Material and Methods section. The amino acid sequences for these constructs can be found on Addgene.
2) We corrected cDNA to plasmid DNA, and the amount of DNA used was added in Material and Methods section.
3) Because electroporation is highly efficient transfection method, we found that 4 hours is enough to express the optogenetic construct and yield an efficient response to photoactivation. The cells begin to appear unhealthy at around 10 hours after electroporation and generally do not survive until the next day, so we perform our imaging assays at 4-10 hours after transfection.

4) We added the laser power measurement in Materials and Methods.

Reviewer 3 Advance Summary and Potential Significance to Field: The authors aim at exploring the mechanisms regulating cell shape. To do so, they use an optogenetic tool that activates RhoA upon blue light stimulation and activate cleavage furrow formation in mononucleated macrophages. This allows them to obtain 2 "half cells", one containing the nucleus and one that does not. They then go on to study the role of FAK and Fascin in regulating cell shape, in particular using cell height and cell/substrate adhesion as a proxy.

Overall, this is an interesting, clear and well written manuscript, using an elegant approach to investigate cell shape with an internal control. However, I do feel that some claims are not sustained by the data, but that in most cases simple quantifications and controls could remedy this. Thus, it is my recommendation that this paper be accepted for publication pending appropriate revisions.

Major:
1. I am a little confused about Figure 2. Are the quantifications in panels D-E performed in FAK overexpressed optogenetically activated cells? Can the authors compare to WT optogenetically activated cells to drive the point that in FAK overexpressed cells the adhesion patterns of the two parts of the cell is driven by FAK? Similarly, how does the height of the part that does contain the nucleus compare with or without FAK overexpression? It seems like the while the height of the non-nucleated half is still smaller than its nucleated counterpart, it is also larger than in controls from figure 1 (which should also be quantified), which suggest that FAK/or FAK mediated adhesion also plays a part in mediating cell height. Could the authors discuss? The authors also claim that there is a higher FAK expression in regions distal to the furrow. This is not obvious from the figure, can this be quantified?
The quantifications in Figure 2D-E were performed on FAK overexpressing cells. Since control cells with wild type levels of FAK are not able to maintain a furrow (Figure 1), they do not generate a nucleated and a non-nucleated side. It is therefore not possible to compare each side of FAK overexpressing cells with the control cells. However, we quantified the relative change in height and adhesion area of the control cells in response to photoactivation (Figure 1E) and showed that neither height not adhesion area exhibited significant change. This shows that the ability of the cells in Figure 2 to maintain the furrow and, importantly, to maintain symmetry in terms of adhesion area, are due to FAK overexpression.
To address the claim that FAK expression is higher in distal regions, we quantified FAK- mCh intensity along a line drawn across the cell (Figure 2F). The graph demonstrates that FAK is indeed localized preferentially to the distal regions.

2. I have the exact same comments on Figure 3. I think the 2 parameters (height, adhesion surface) should be compared to control cells. Otherwise, the authors can only conclude about the role of fascin (or FAK in the case of Figure 2) in the differential shapes between nucleated and non nucleated, but cannot make general claims about the role of fascin or FAK on cell shape. The authors should also quantify their claim that fascin expression is higher in the non nucleated part (using a Z-plan that does not contain the nucleus). In particular, in this case, it is important to look at fascin underlying the actin cortex, since it is doubtful fascin in the cytoplasm plays any part.

As mentioned above, the nucleated and non-nucleated sides do not exist in control cells, making a direct comparison with fascin-overexpressing sides impossible. However, the finding that fascin-overexpressing cells are able to maintain the furrow and generate two sides with similar heights demonstrates fascin's role in maintaining height while the cell undergoes shape changes.
We quantified the fascin-mCh distribution along a line drawn across the cell (Figure 3F), showing that fascin is present in higher amounts in the non-nucleated side. Since this significant increase in fascin in the non-nucleated side results in the cell height being similar to the nucleated side, we infer that regardless of the specific intracellular distribution, fascin plays a role in determining cell height.

3. Figure 4C, it seems like RhoA activation in Fascin overexpressed cells leads to an elongation of the cell, possibly by spreading on the substrate. Is that true? Could the authors quantify this?

We assume that the Reviewer is referring to Figure 3C, which shows that fascin- overexpressing cells are able to form a furrow, characterized by RhoA-mediated contraction in the middle and formation of two halves. This has all the characteristics of furrow formation during cytokinesis as we have reported earlier (Castillo-Badillo et al., 2020). The two sides of the cell moving apart resembles cell elongation.

4. Line 371: the authors claim that the persistence of cell shape changes are due to higher level of FAK maintaining a greater adhesion. This is not clear from the data. The authors should test this by repeating the experiment on micropatterns of different adhesion (either modulating the strength of the adhesion via coating eg https://www.nature.com/articles/s41467-020-20563-9, or maybe the size of the adhesion pattern).

In response to this comment, we used TIRF imaging to visualize adhesion dynamics during cell shape changes. We quantified the fluorescence intensity of membrane marker Venus-KRasCT in the TIRF plane, since the proximity of the membrane at the substrate is indicative of adhesions. We found that FAK-overexpressing cells indeed exhibit dramatically higher Venus-KRasCT fluorescence intensity in the TIRF plane compared to control cells or fascin overexpressing cells (Figure 4A-C).

5. Induction of changes in local contractility leading to a furrow-like structure in mononucleated cells cannot be directly compared to the processes involved in controlling cell shape during mitosis. Despite the absence of nucleus and a characteristic organisation of the actin network, other cytoskeletal components important for maintaining cell shape drastically reorganise during cell division, impact cell surface mechanics and affect cell shape. While the findings of the study can be used to infer the potential mechanisms controlling cell shape during mitosis, the authors should not use the direct comparison. The phenotype observed in this study might be more relevant for the study of amoebal migration then cell division. Therefore, the authors should tone down their claim that this model allows a direct comparison with mitosis.
In a side note, the data in Movie S1, Figure 1 and Movie S5 and Figure 5 suggests that the system presented could be an interesting model of migration. Therefore, quantifying data related to migration (such as speed and direction) upon RhoA photoactivation might offer interesting insights about mechanisms for migration. This is beyond the scope of this paper but could be interesting in the future.

As suggested by the reviewer we have not discussed the findings here being directly relevant to mitosis.
We thank the reviewer for thoughtful suggestions for future experiments where we could apply this methodology to studying migration. We have in the past pursued this to some extent (O'Neill et al 2018; Meshik et al 2019).

6. The authors should change the bar graphs to dot plots, to be more transparent about the number of points, especially since the authors use very small n numbers and SEM instead of SD. In the few quantified experiments, the n number is pretty low, and the paper would benefit from adding some data points if possible (and in particular, the experiments presented in figure 1 should be quantified, and SD should be used over SEM for n=45).

As mentioned above, we obtained z-stacks for a subset of cells due to experimental challenges associated with single cell optogenetics experiments. Since the bars allow easier visualization of differences we retained them. Since the experiments were performed independently over multiple days we used SEM.

We also performed additional quantification of FAK and fascin polarization and quantified the height and adhesion area changes in control cells (Figure 1E).

Minor:
7. In the introduction, the authors suggest that the existing experimental methods to study cell shape changes are limited to growing cells on different surfaces ignoring a variety of confinement methods. Manipulation of cell shape through more biochemical approaches of depletion, overexpression or use of inhibitors is also ignored. Since the optogenetic approach induces cell shape through activation of RhoA and thus signalling, more traditional biochemical approaches

are also relevant. The authors should amend the text to not ignore this body of literature.

We agree with the reviewer that it is important to acknowledge this body of literature. We have now referred to these methods and cited additional references in the Introduction.

8. Line 181: it should be clear that FAK is overexpressed in these cells. Could the authors quantify the amount of overexpression by western blot?

One of the advantages of using fluorescently-tagged proteins is that we can see their expression in a single cell in real time. We can study their distribution, dynamics, and polarization, depending on the cell behavior. This presents a distinct advantage in live cell studies over Western blotting of a pool of cells.

9. Line 371: the authors say that the role of fascin in maintaining cell height is counterintuitive. I would argue that published in vitro and in silico data suggest differently (Chugh at al., 2017; Ennomani et al., 2016; Ding et al., 2017). Fascin is an actin crosslinker: more crosslinking will lead to a higher tension and thus a rounding of the cells.
Furthermore, there are multiple different actin bundling proteins present in the cells. The selection of fascin over more commonly used alpha-actinin 4 is somewhat surprising and I believe authors should explain why they chose this particular actin bundler. Additional discussion and data with other actin bundlers might also confirm whether the observed mechanisms is due to the bundling activity of fascin. The authors might also be interested in findings by Logue et al., 2015 where actin bundler Eps8 is shown to affect migration. Since the model is applied to a cell division, Dix et al. paper could be discussed in relation with focal adhesion part of the model.

We decided to focus on fascin because of its known role in membrane and nuclear *shape and in maintaining cytoskeletal rigidity. We agree that other actin bundling proteins can also play a role in this process and we have modified the discussion to reflect this.*

10. The discussion should be toned down, it should be clear where the authors are hypothesising. For ex line 439-442, the authors have not shown that FAK is more recruited distally, and they have not shown that the bottom of the cell is more stable. If they want to claim this, then they should show that it is more stable, for example by showing and quantifying kymographs of the plasma membrane in that bottom plane. Similarly, line 461-462, it is not clear that fascin is present in higher quantities on the side without a nucleus, as it has not been properly quantified.

To address this, we quantified the distribution of FAK and fascin across the length of the cell (Figures 2F and 3F). This showed that FAK is indeed present in greater amounts in distal regions of the cell, whereas fascin is preferentially polarized to the non-nucleated side. To demonstrate greater adhesion of membrane to the substrate in FAK- overexpressing cells, we performed TIRF experiments visualizing adhesions in control, fascin, and FAK overexpressing cells (Figure 4). We found that FAK overexpressing cells exhibit dramatic increase in adhesion compared with the control cells or fascin overexpressing cells.

11. The authors just justify the use of a t test (by at least saying their data follows a normal distribution). Furthermore, if they have n=42 cells (as presented in 2A for example), they should analyse 42 cells and not 11 (as presented in 2D for example) or justify why they cannnot.

Our data did follow a normal distribution, and we added a mention of it to the Materials and Methods.
As mentioned above, we imaged 46 cells in a single confocal plane, but due to challenges associated with single cell optogenetics experiments, obtained z-stack for 11 of those cells.

12. A few typos:
line 81: Repeated punctuation

line 113: Missing reference for regulation of FAK and fascin by RhoA. line 142: mono-nucleated should be mononucleated
We corrected the typos.

CASTILLO-BADILLO, J. A., BANDI, A. C., HARLALKA, S. & GAUTAM, N. 2020. SRRF-Stream Imaging of Optogenetically Controlled Furrow Formation Shows Localized and Coordinated Endocytosis and Exocytosis Mediating Membrane Remodeling. *ACS Synth Biol,* 9, 902- 919.
GOLUBOVSKAYA, V. M., NYBERG, C., ZHENG, M., KWEH, F., MAGIS, A., OSTROV, D. & CANCE, W. G. 2008. A small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, targeting the y397 site of focal adhesion kinase decreases tumor growth. *J Med Chem,* 51, 7405-16.
HUANG, F. K., HAN, S., XING, B., HUANG, J., LIU, B., BORDELEAU, F., REINHART-KING, C. A., ZHANG, J. J. & HUANG, X. Y. 2015. Targeted inhibition of fascin function blocks tumour invasion and metastatic colonization. *Nat Commun,* 6, 7465.

Second decision letter

MS ID#: JOCES/2020/258321

MS TITLE: Optogenetic Model Reveals Cell Shape Regulation Through FAK and Fascin

AUTHORS: Jean A Castillo-Badillo and N Gautam
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, reviewers #2 found that you have satisfactorily addressed his/her comments and recommends publication. However, reviewer # 1 and 3 still have some important issues that you will need to address before submitting your final manuscript for publication. These concern (1) the inappropriate use of statistics (2) the lack of quantitative data (3) the use of bar graphs over dot plots and (4) validation of the effects of the FAK inhibitor. I trust that you will be able to properly deal with them and look forward to receiving a further revision of your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

This study utilizes an optogenetic tool to activate RhoA in the center of a mononucleated macrophage and induce contraction, thereby causing changes in cell shape that are associated with

cleavage furrow formation. This manipulation allows for the study of mechanisms involved in cell shape maintenance during furrow formation of nascent daughter cells, which lack the mechanical support provided by a nucleus. The authors use the model to study how the RhoA effectors, FAK and fascin, each contribute to maintenance of cell shape in the absence of nuclear support. If the authors can improve the rigor of the experiments that use pharmacological inhibitors to support their model, then the study's impact will be greatly improved.

Comments for the author

My previous concern remains that the experiments using pharmacological inhibitors of FAK (FAK14i) and fascin (G2) were not performed with sufficient rigor to support the authors' conclusions. In response to this concern, the authors state that these inhibitors have been "extensively characterized" in the literature, but they cite a single paper - Golubovskaya et al. (2008) - to support their use of 10 μM FAK14i to inhibit FAK phosphorylation. Importantly, the latter study used cancer cells, while the current study uses macrophages.

FAK expression levels/activity can vary between cell types, such that different inhibitor concentrations may be required. The authors should show that the inhibitor works as expected in macrophages under the conditions used, rather than rely on an earlier study in a different cell type.

In their response, the authors claim that "extensive characterization of this inhibitor has demonstrated via western blotting that FAK14i inhibits FAK phosphorylation at site Y397 with high specificity….." However, a recently published paper (Dayang et al., 2021, PMID:33378972) shows that treatment of endothelial cells with FAK14i did not reduce FAK auto-phosphorylation, although it consistently inhibited an inflammatory phenotype. Dayang et al. concluded that the anti-inflammatory effect of FAK14i was independent of Y397 phosphorylation, even pointing out that their findings are in contrast with those of Golubovskaya et al. (2008). Moreover, Dayang et al. demonstrated non-specific effects of FAK14i in their model. Indeed targeting FAK with siRNA versus treating with FAK14i produced differences in phenotypes, which they suggest were due to non-specific effects of FAK14i on other kinases (i.e., Pyk2).

In my opinion, it is important that the authors to validate the effects of FAK14i in their own model to support their conclusion about FAK's key involvement. They respond that RNAi may have secondary effects that may cloud interpretation, but this is also true of pharmacological inhibitors such as FAK14i (e.g., Dayang et al., 2021). At a minimum, they should provide western blots that FAK14i effectively inhibits FAK phosphorylation in their macrophage model as they claim (line 277), and provide an alternative explanation if it does not.
These experiments are not difficult to do and provide a standard level of rigor when using pharmacological inhibitors.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all my comments/concerns so that I now can support publication of the manuscript.

Comments for the author

The authors have addressed all my comments/concerns so that I now can support publication of the manuscript.

Reviewer 3

Advance summary and potential significance to field

The authors aim at exploring the mechanisms regulating cell shape. To do so, they use an optogenetic tool that activates RhoA upon blue light stimulation and activate cleavage furrow formation in mononucleated macrophages. This allows them to obtain 2 “half cells”, one containing the nucleus and one that does not. They then go on to study the role of FAK and Fascin in regulating cell shape, in particular using cell height and cell/substrate adhesion as a proxy.
As I said in my first assessment, this is an interesting manuscript, using an elegant approach to investigate cell shape with an internal control. However, my concerns have not been addressed by the authors. In particular, some claims are not sustained by the data, and the lack of controls and inappropriate use of statistics and plots is both weakening the claims and making the rational of the paper unclear. Thus, it is my recommendation that this paper be accepted for publication pending appropriate revisions.

Comments for the author

1) Comparisons between the nucleated and non nucleated sides. This relates to my previous comments (pasted below with the author response). If the authors do not want to compare those 2 sides on the claim that there is no fixed shape on the non nucleated side, then I do not understand the point of the study.

The difference in the adhesion area and height between WT and cells with overexpression could at least be measured at t=0 to show the role of the overexpression on the cell shape before any of the treatments.

Additionally, the authors do not show which part of the cell was used to draw the line for the quantification of the FAK/fascin expression. The results might differ based on the part of the cell used for this quantification. Since there is no evidence of the amount of overexpressed protein compared to endogenous (which could be addressed with suggested Western blots), it is impossible to conclude whether these differences in localization are mechanistically important or the artifact of overexpression.

Figure 1E: It is not entirely clear how the comparison of the two states was made. I am assuming that the cell was measured at the beginning and the end and for each cell these two measurements were used to obtain relative value. These details of analysis should be clearly stated. Also, the legend doesn’t state if SD or SEM is plotted.

My previous comments and the authors reponse: I am a little confused about Figure 2. Are the quantifications in panels D-E performed in FAK overexpressed optogenetically activated cells? Can the authors compare to WT optogenetically activated cells to drive the point that in FAK overexpressed cells the adhesion patterns of the two parts of the cell is driven by FAK? Similarly, how does the height of the part that does contain the nucleus compare with or without FAK overexpression? It seems like the while the height of the non-nucleated half is still smaller than its nucleated counterpart, it is also larger than in controls from figure 1 (which should also be quantified), which suggest that FAK/or FAK mediated adhesion also plays a part in mediating cell height. Could the authors discuss? The authors also claim that there is a higher FAK expression in regions distal to the furrow. This is not obvious from the figure, can this be quantified?

The quantifications in Figure 2D-E were performed on FAK overexpressing cells. Since control cells with wild type levels of FAK are not able to maintain a furrow (Figure 1), they do not generate a nucleated and a non-nucleated side. It is therefore not possible to compare each side of FAK overexpressing cells with the control cells. However, we quantified the relative change in height and adhesion area of the control cells in response to photoactivation (Figure 1E) and showed that neither height not adhesion area exhibited significant change. This shows that the ability of the cells in Figure 2 to maintain the furrow and, importantly, to maintain symmetry in terms of adhesion area, are due to FAK overexpression.

To address the claim that FAK expression is higher in distal regions, we quantified FAK-mCh intensity along a line drawn across the cell (Figure 2F). The graph demonstrates that FAK is indeed localized preferentially to the distal regions.
2. I have the exact same comments on Figure 3. I think the 2 parameters (height, adhesion surface) should be compared to control cells. Otherwise, the authors can only conclude about the role of

fascin (or FAK in the case of Figure 2) in the differential shapes between nucleated and non nucleated, but cannot make general claims about the role of fascin or FAK on cell shape. The authors should also quantify their claim that fascin expression is higher in the non nucleated part (using a Z-plan that does not contain the nucleus). In particular, in this case, it is important to look at fascin underlying the actin cortex, since it is doubtful fascin in the cytoplasm plays any part.
As mentioned above, the nucleated and non-nucleated sides do not exist in control cells, making a direct comparison with fascin-overexpressing sides impossible. However, the finding that fascin-overexpressing cells are able to maintain the furrow and generate two sides with similar heights demonstrates fascin's role in maintaining height while the cell undergoes shape changes.
We quantified the fascin-mCh distribution along a line drawn across the cell (Figure 3F), showing that fascin is present in higher amounts in the non-nucleated side. Since this significant increase in fascin in the non-nucleated side results in the cell height being similar to the nucleated side, we infer that regardless of the specific intracellular distribution, fascin plays a role in determining cell height.
2) quantifications: the authors still make claims that are not supported by the data. For example
- line 143: "We subsequently refer to these cells as control cells because they express wild type levels of FAK and fascin." This has not been demonstrated and should be quantified. I am happy to see a quantification of immunofluorescence images rather than western blot as I suggested, since the authors say in their response: "One of the advantages of using fluorescently-tagged proteins is that we can see their expression in a single cell in real time. We can study their distribution, dynamics, and polarization, depending on the cell behavior. This presents a distinct advantage in live cell studies over Western blotting of a pool of cells." But there need to be a quantification if there is such a claim.
- 4. Line 371: the authors claim that the persistence of cell shape changes are due to higher level of FAK maintaining a greater adhesion. This is not clear from the data. I suggested "The authors should test this by repeating the experiment on micropatterns of different adhesion (either modulating the strength of the adhesion via coating eg https://www.nature.com/articles/s41467-020-20563-9, or maybe the size of the adhesion pattern)."

The authors say "In response to this comment, we used TIRF imaging to visualize adhesion dynamics during cell shape changes. We quantified the fluorescence intensity of membrane marker Venus-KRasCT in the TIRF plane, since the proximity of the membrane at the substrate is indicative of adhesions. We found that FAK-overexpressing cells indeed exhibit dramatically higher Venus-KRasCT fluorescence intensity in the TIRF plane compared to control cells or fascin overexpressing cells (Figure 4A-C).".

This is also a correlation and still not a demonstration. I think this is an interesting observation, but again, the authors should tone done down their claim or demonstrate causality.
Furthermore, if the authors decide to show the TIRF data they should address the significance of the decrease in the maximum TIRF intensity in fascin overexpressed cells compared to wild type.
2) The use of bar graph over dot plots does not allow "a better visualisation" of the data. Dot plots are more transparent because they allow to see the n numbers and distribution of the data. The only case where this is justified is things such as contingency plots where the results are shown are part of a whole over a whole experiment, which is not the case here. In this manuscript in particular, the authors show some n number and then only quantify a subset of data because of "experimental challenges". While I am not certain what those challenges are (if the authors can claim that the image is representative of 45 cells, it is difficult to understand while Z-stacks cannot be acquired for only 11 cells), we should at least be able to see the 11 points on the graph and have an idea of the spread of the data. Furthermore, if in the statistics individual cells are compared rather than individual experiments, SD is more appropriate than SEM. Furthermore, while the methods now state the distribution is normal there is no mention of how this was assessed. Without the individual data points plotted, it's impossible to make a visual estimate of the legitimacy of the claim.

3) statistics: why are the t-tests paired? A paired t-test is useful when each cell is measured twice, so in this case it would mean that the cell is measured before activation and after. However given how the experiments are described, it does not seem to be the case.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

They should provide western blots that FAK14i effectively inhibits FAK phosphorylation in their macrophage model as they claim (line 277) and provide an alternative explanation if it does not. These experiments are not difficult to do and provide a standard level of rigor when using pharmacological inhibitors.

We agree with the reviewer especially because as pointed out it will improve the rigor of the paper. As suggested, we tested the inhibitor FAKi14 by western blotting in RAW 264.7 macrophages. We transfected the cells with the optogenetic construct and after allowing time for expression, treated the cells for 30 min with 10 μM inhibitor, FAKi14. The cells were then globally photoactivated for 15 min. This activates RhoA and as a result FAK. We found that FAKi14 was able to inhibit the phosphorylation of Tyr397 in FAK in comparison to control. This confirms that FAKi14 is inhibiting FAK phosphorylation as described in our model. The western blot results are provided in Supplemental Fig 1.

Reviewer 2 Comments for the Author:

The authors have addressed all my comments/concerns so that I now can support publication of the manuscript.

Reviewer 3 Comments for the Author:

The difference in the adhesion area and height between WT and cells with overexpression could at least be measured at t=0 to show the role of the overexpression on the cell shape before any of the treatments.

As mentioned in the manuscript, FAK and fascin are both downstream of RhoA. Therefore, the effect of their overexpression is expected to be only observed after RhoA activation. It is therefore expected that there will be no difference in height or adhesion area between wild type cells and cells overexpressing FAK or fascin at t=0. As anticipated, there was no difference. We have presented these results in Supplemental Figure 2.

Additionally, the authors do not show which part of the cell was used to draw the line for the quantification of the FAK/fascin expression.

Fig 2F and 3F show a scheme with the intensity profile line. This line connects the distal edges of the cell and crosses the furrow.

Figure 1E: It is not entirely clear how the comparison of the two states was made. I am assuming that the cell was measured at the beginning and the end and for each cell these two measurements were used to obtain relative value. These details of analysis should be clearly stated. Also, the legend doesn't state if SD or SEM is plotted.

This is correct, we measured height and adhesion area at beginning and end and took the ratio to obtain the relative change after photoactivation. This is described in the Fig legend. The plot now shows the standard deviation, and we changed the bar graphs for dot plots, like the reviewer suggested.

line 143: "We subsequently refer to these cells as control cells because they express wild type levels of FAK and fascin." This has not been demonstrated and should be quantified. I am happy to see a quantification of immunofluorescence images rather than western blot as I suggested, since the authors say in their response: "One of the advantages of using fluorescently-tagged proteins is that we can see their expression in a single cell in real time. We can study their distribution, dynamics, and polarization, depending on the cell behavior. This presents a distinct advantage in

live cell studies over Western blotting of a pool of cells." But there need to be a quantification if there is such a claim.

We use the phrase "wild type levels" because we did not transfect the control cells with FAK or fascin. This term is used widely to denote cells expressing native levels of proteins. Immunoblotting will not provide an accurate measure of protein expression in these cells as they are transfected and express different levels. Immunofluorescence will not be appropriate since the cells are being studied here in the living state for studying a dynamic behavior. The presence of mCherry fluorescence demonstrates the presence of expressed proteins in the cell. We have quantified the mCherry fluorescence intensity profile across the cell for FAK and fascin, as shown in Fig 2F and 3F.

Line 371: the authors claim that the persistence of cell shape changes are due to higher level of FAK maintaining a greater adhesion. This is not clear from the data. I suggested "The authors should test this by repeating the experiment on micropatterns of different adhesion (either modulating the strength of the adhesion via coating eg https://www.nature.com/articles/s41467-020-20563-9, or maybe the size of the adhesion pattern)."
The authors say "In response to this comment, we used TIRF imaging to visualize adhesion dynamics during cell shape changes. We quantified the fluorescence intensity of membrane marker Venus-KRasCT in the TIRF plane, since the proximity of the membrane at the substrate is indicative of adhesions. We found that FAK-overexpressing cells indeed exhibit dramatically higher Venus-KRasCT fluorescence intensity in the TIRF plane compared to control cells or fascin overexpressing cells (Figure 4A-C)." This is also a correlation and still not a demonstration. I think this is an interesting observation, but again, the authors should tone done down their claim or demonstrate causality.

While micropatterns can be useful for visualizing static adhesions, as was done in the paper mentioned by the reviewer, we think TIRF experiments are the more appropriate method for identifying dynamic changes in a live cell where both cellular and molecular changes need to be monitored in real time at the adhesion surface. The evanescent wave restricts imaging to less than 100 nm so it is well suited to provide high resolution images of a narrow area at the adhesion surface. We therefore feel that this is the appropriate technique to monitor adhesion dynamics over time in these cells. TIRF experiments have been used before for this purpose (Berginski et al., 2011, Stehbens and Wittmann, 2014, Le Devedec et al., 2010).
We suggest that adhesion turnover is a mechanism that is involved in cell shape maintenance through a process of detachment and attachment of the cell. Since FAK is downstream of RhoA, RhoA activation results in increased FAK phosphorylation, as shown in the western blot in Supplemental Fig 1. We conclude that the increased KRasCT in the TIRF plane demonstrates increased adhesions in response to FAK activation. Viewed together with the other evidence we have provided here on a role for FAK in cells shape determination, our suggestion that FAK plays a role in cells shape determination is not solely based on this result but together with other evidence provided in Figure 2 and Figure 5. As suggested by the Reviewer we have discussed this appropriately in the Discussion.
The fluorescent intensity of KRasCT in the TIRF images in fascin overexpressed cells compared to wild type is not statistically significant (Fig 4D).

Dot plots are more transparent because they allow to see the n numbers and distribution of the data. The only case where this is justified is things such as contingency plots where the results are shown are part of a whole over a whole experiment, which is not the case here. In this manuscript in particular, the authors show some n number and then only quantify a subset of data because of "experimental challenges". While I am not certain what those challenges are (if the authors can claim that the image is representative of 45 cells, it is difficult to understand while Z-stacks cannot be acquired for only 11 cells), we should at least be able to see the 11 points on the graph and have an idea of the spread of the data. Furthermore, if in the statistics individual cells are compared rather than individual experiments, SD is more appropriate than SEM.

As suggested, we have replaced the bar graphs with dot plots for a better visualization and transparency of our data. We also show the SD, as suggested. This is now mentioned in the Fig

legends.
To our knowledge no other laboratory other than ours performs Z stacking of live cells that are optogenetically controlled to execute specific dynamic cellular behaviors such as cell shape changes or migration. These are complex and challenging experiments. Even the very limited overlap in the wavelengths that are used to acquire the serial confocal images that provide a Z stack and the light sensitive proteins that are part of the optogenetic tool result in global activation. As a consequence, the cell stops responding. Given this, we think it is reasonable to infer that the results from a fairly large number of cells are representative of a bigger population.

Statistics: why are the t-tests paired? A paired t-test is useful when each cell is measured twice, so in this case it would mean that the cell is measured before activation and after. However, given how the experiments are described, it does not seem to be the case.

We used the paired t-test when comparing measurements of two sides of the same cell (the nucleated side and the non-nucleated side, Figs 2D-E, 3D-E). This is an appropriateuse of the paired t-test. When comparing different cell populations, we used a two-sample t-test (Fig 4D). The type of statistical analysis used is mentioned in the Fig legends.

Third decision letter

MS ID#: JOCES/2020/258321

MS TITLE: Optogenetic Model Reveals Cell Shape Regulation Through FAK and Fascin

AUTHORS: Jean A Castillo-Badillo and N Gautam
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.
Reviewer 3

Advance summary and potential significance to field

The authors aim at exploring the mechanisms regulating cell shape. To do so, they use an optogenetic tool that activates RhoA upon blue light stimulation and activate cleavage furrow formation in mononucleated macrophages. This allows them to obtain 2 “half cells”, one containing the nucleus and one that does not. They then go on to study the role of FAK and Fascin in regulating cell shape, in particular using cell height and cell/substrate adhesion as a proxy.

Comments for the author

The authors have no answered all of my concerns and I am happy for the paper to be published.