Emerin regulation of nuclear stiffness is required for fast amoeboid migration in confined environments

Sandrine B. Lavenus, Karl W. Vosatka, Alexa P. Caruso, Maria F. Ullo, Ayesha Khan and Jeremy S. Logue
DOI: 10.1242/jcs.259493

Editor: Michael Way

Review timeline
Original submission: 20 October 2021
Editorial decision: 29 November 2021
First revision received: 17 February 2022
Editorial decision: 15 March 2022
Second revision received: 16 March 2022
Accepted: 17 March 2022

Original submission

First decision letter

MS ID#: JOCES/2021/259493

MS TITLE: Emerin regulation of nuclear stiffness is required for fast amoeboid migration in confined environments

AUTHORS: Sandrine B Lavenus, Karl W Vosatka, Maria F Ullo, and Jeremy S Logue
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 raise a number of substantial criticisms that prevent me from accepting the paper at this stage. However, I think that a revised version might prove acceptable, if you can address their concerns, which largely consist of additional quantification and controls. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

With this manuscript, Lavenus and colleagues continue investigate a very interesting form of single cell migration through confined environments, namely fast leader bleb-based amoeboid migration. This form of migration requires the cells to be confined and unable to use integrin-based adhesion with the surrounding matrix/confining surfaces. They are specifically interested in learning how the rigidity of the nucleus is regulated and how that physical change in turn dictates the speed of fast amoeboid migration. This work builds on the work of the Logue lab, as well as other groups working on amoeboid migration, as well as other modes of 3D cell movement. The authors conclude here that Src family kinase phosphorylation of emerin regulates lamin A to control the degree of nuclear envelope stretch. The greater the stretch of the nuclear envelope triggers cPLA2 activation to increase actomyosin contractility at the plasma membrane to increase the speed and percentage of cells using fast amoeboid migration.

This straight forward and logical pathway is not strongly supported by the data presented. The paper suffers from a lack of controls and rigorous quantification. It seems that the relationship between the expression of emerin and lamin A do not always affect nuclear stiffness in the predicted way, and when there are changes in stiffness, they don't always support the model presented. I think the fundamental issue is the paper is too focused on proving a reasonable and logical hypothesis, and that the authors overlook potential alternative explanations based on the data presented.

Comments for the author

It is not clear in the final paragraph of the introduction what is being described in this paper versus previously published works.
Are the cells using bleb-based migration to get through the transwell pores in the 1C' migration assay?
Many controls and quantification are missing from the paper. For example, no independent siRNA sequences are used to confirm that the phenotypes are not due to off target effects and the degree of overexpression of exogenous proteins is not quantified. Further, there is no indication that equivalent expression levels were compared between experiments.
siRNA western blots are in the supplemental but not referred to in the text.
Five supplemental movies are included, but they are also not referred to in the text. Obviously if the data is important enough to include in the manuscript, it should be referred to.
The first paragraph comes across as a collection of observations that the authors then assume will make sense to the reader and they can follow the logic.
In fact, it is not at all clear how these observations support the preliminary conclusions stated by the authors.
The difference between control and lamin a OE in Figure 1D are quite small. It would be helpful to include representative images either in the main figure or as a supplemental.
I question the utility of the conceptual figures (1k, 2j, and 4m). I don't think the data presented fully supports these conceptual relationships and it comes across as wishful thinking or potentially misleading. This type of conceptualization should be restricted to the discussion.
Should incorporate lack of adhesion into the hypothesized pathways (Figure 1B for example)? How would integrin-based adhesion change the outcome of these pathways?
What is the significance of the nucleus being in the leader bleb?
Why would emerin siRNA lead to rounder nuclei? Should they not be more easily deformed?
For figure 3, it is really not clear the logic of treating with LA, or why nuclear F-actin is mentioned. It heavily implies that nuclear stiffness is not measured in these cells. I question then, why is the data included as nuclear stiffness?

I am not convinced based on the imaging data presented that emerin delta INM is not still in the nuclear envelope. Can this shift be quantified somehow?
Major question: Does this cell type actually have the lamin A/C nuclear stiffening mechanism?
Is figure 4I different data then that presented in Figure 3G? If it is the same that should be explicitly stated in the paper.
The FRET experiments are also missing key controls as well as indications of how equivalent levels of reporter expression were imaged in the different experiments. At a minimum, they should show that treating cells expressing the reporter with a Src inhibitor has the expected effect on the FRET signal.

Reviewer 2

Advance summary and potential significance to field

The work by Lavenus et al. adds new important mechanistic data supporting the model in which the nucleus non-transcriptionally converts mechanical cues into chemical signals impacting cell morphodynamics in mechanically restrictive microenvironments. The authors present a series of experimental manipulations and quantitative analyses to illuminate a rather nonintuitive feature of biophysical processes involved in amoeboid cell motility and cell shape control which is nonlinearity. Indeed, based on their experiments, Lavenus et al. show that interfering with the ability of the nuclear envelope to be stretched in response to cellular compression affects blebbing cell morphodynamics.
Specifically, the authors show that emerin-dependent modulation of nuclear stiffness plays a decisive role: nuclear stiffness that is either too small or too high is detrimental to stretching of the nuclear envelope, suggesting that an optimal nuclear envelope stiffness should be maintained in order for the nucleus to be stretchable. Lavenus et al. propose that emerin-dependent control of nuclear envelope stretchability can affect the activity of the nuclear stretch-sensitive protein cPLA2, which in turn impacts the signaling to the actomyosin cortex and blebbing cell morphodynamics. I am curious whether the authors can provide any experimental evidence in support of this proposal. One can think of a simple experiment in which the low contractility/blebbing phenotype downstream of emerin-dependent modulation of nuclear stiffness (and thus cPLA2 recruitment to nuclear/perinuclear ER membranes) can be rescued by the addition of (1) ionomycin to artificially boost levels of intracellular Ca2+
allosterically activating cPLA2 and/or (2) purified arachidonic acid – the product of cPLA2's enzymatic activity. I believe these experiments are relatively easy to perform within a reasonable time frame and their results may add to the completeness of the story, therefore reinforcing its conclusions.

Comments for the author

The work by Lavenus et al. adds new important mechanistic data supporting the model in which the nucleus non-transcriptionally converts mechanical cues into chemical signals impacting cell morphodynamics in mechanically restrictive microenvironments. The authors present a series of experimental manipulations and quantitative analyses to illuminate a rather nonintuitive feature of biophysical processes involved in amoeboid cell motility and cell shape control which is nonlinearity. Indeed, based on their experiments, Lavenus et al. show that interfering with the ability of the nuclear envelope to be stretched in response to cellular compression affects blebbing cell morphodynamics.
Specifically, the authors show that emerin-dependent modulation of nuclear stiffness plays a decisive role: nuclear stiffness that is either too small or too high is detrimental to stretching of the nuclear envelope, suggesting that an optimal nuclear envelope stiffness should be maintained in order for the nucleus to be stretchable. Lavenus et al. propose that emerin-dependent control of nuclear envelope stretchability can affect the activity of the nuclear stretch-sensitive protein cPLA2, which in turn impacts the signaling to the actomyosin cortex and blebbing cell morphodynamics. I am curious whether the authors can provide any experimental evidence in support of this proposal. One can think of a simple experiment in which the low contractility/blebbing phenotype downstream of emerin-dependent modulation of nuclear stiffness (and thus cPLA2 recruitment to nuclear/perinuclear ER membranes) can be rescued by the addition of (1) ionomycin to artificially boost levels of intracellular Ca2+ allosterically activating cPLA2

and/or (2) purified arachidonic acid – the product of cPLA2's enzymatic activity. I believe these experiments are relatively easy to perform within a reasonable time frame and their results may add to the completeness of the story, therefore reinforcing its conclusions.

Reviewer 3

Advance summary and potential significance to field

- In this interesting manuscript, authors show that Emerin regulates leader bleb formation in cells migrating under confinement. This study adds to the breath of knowledge accumulating with regards to nuclear dynamics in blebbing cells migrating under confinement.

Comments for the author

Authors suggest that levels of these proteins affect migration. The data suggests that migration speed of all cells is reduced because a smaller percentage of cells now adopt the leader mobile (LM) phenotype. Migration of LM cells themselves is not altered (Fig 1H, Fig 2G, Fig 4G). As such, Lamin A/ Emerin are upstream regulators of bleb dynamics, but I am not sure I would conclude they impair migration per se. Maybe some simple rephrasing would be required.
On the other hand, as part of their mechanism, authors argue that there are changes in actomyosin contractility driving migration, but all they quantify are changes in leader bleb formation. To prove actomyosin contractility is involved I would like to see some quantification of MLC2 phosphorylation levels and/or careful analysis of pMLC2 and actin localisation. In Supp Fig 4 F-actin levels are measured by FACS but more careful assessment of localisation is required.
- Levels of Lamin A and Emerin after knockdown and over-expression should be shown by wb and/or ideally at single cell level. I think this is required to validate their proposed mechanisms. An attempt was made in Fig 1J, and shows quite a spread of over-expression. Authors could over-express different levels of Lamin A and Emerin to show it is a concentration dependent effect. In those lines, are all the emerin constructs expressed to the same level? A western blot would help
- Some quantification of cell viability is also required. Manipulating the nuclear envelope can have detrimental effects on cells so it would be good to confirm how 'healthy' these cells are.
- The graphs with % cells (Fig 1E, Fig 2D,F, Fig 4F) do not have any stats or error bars on them. These should be provided.
- Are all the experiments carried out with the 3 micron confinement? This could be reiterated in some of the figure legends for clarity. If authors are using 3 micron confinement for most of the work, transwell assays in Fig 1 should also use pores of that size rather than 8 micron. Authors have only used a cell line, A375M2 metastatic melanoma line, with very high levels of intrinsic actomyosin. Could they repeat key experiments to see what happens with a cell line with low actomyosin levels to start with, does it form less LM?

First revision

Author response to reviewers' comments

Author responses are in red

Reviewer 1

Advance Summary and Potential Significance to Field:

With this manuscript, Lavenus and colleagues continue investigate a very interesting form of single cell migration through confined environments, namely fast leader bleb-based amoeboid migration. This form of migration requires the cells to be confined and unable to use integrin-based adhesion with the surrounding matrix/confining surfaces. They are specifically interested in learning how the rigidity of the nucleus is regulated and how that physical change in turn

dictates the speed of fast amoeboid migration. This work builds on the work of the Logue Lab, as well as other groups working on amoeboid migration, as well as other modes of 3D cell movement. The authors conclude here that Src family kinase phosphorylation of emerin regulates Lamin A to control the degree of nuclear envelope stretch. The greater the stretch of the nuclear envelope triggers cPLA2 activation to increase actomyosin contractility at the plasma membrane to increase the speed and percentage of cells using fast amoeboid migration. This straightforward and logical pathway is not strongly supported by the data presented. In order to de-emphasize the proposed pathway, it has been removed from most of the figures. We also discuss alternative mechanisms. The paper suffers from a lack of controls and rigorous quantification. It seems that the relationship between the expression of emerin and Lamin A do not always affect nuclear stiffness in the predicted way, and when there are changes in stiffness, they don't always support the model presented. I think the fundamental issue is the paper is too focused on proving a reasonable and logical hypothesis, and that the authors overlook potential alternative explanations based on the data presented.

Reviewer 1

Comments for the Author:

1. It is not clear in the final paragraph of the introduction what is being described in this paper versus previously published works.

We apologize for this, we have now included an additional paragraph that summarizes what is being described in this paper to the end of the Introduction.

2. Are the cells using bleb-based migration to get through the Transwell pores in the 1C' migration assay?

Unfortunately, we do not have enough information to make any claims about the mode of migration used by melanoma cells through filters. As the deformability of the nucleus can in general regulate migration through confined environments, we have used filters with differing pore sizes as a general approach for measuring rates of motility. Transmigration assays have been used in this way previously, such as in Lomakin, A *et al*. (2020) *Science*.

3. Many controls and quantification are missing from the paper. For example, no independent siRNA sequences are used to confirm that the phenotypes are not due to off target effects and the degree of overexpression of exogenous proteins is not quantified. Further, there is no indication that equivalent expression levels were compared between experiments.

We have included additional data (figure 2J), which demonstrates that defects in transmigration through 8 µm pores after RNAi can be rescued with an RNAi resistant version of emerin. Moreover, a cell-by-cell analysis (i.e., immunofluorescence), was conducted in figures 1H, 2H, 4L, S2I, and S3H, demonstrating that equivalent levels of emerin WT and mutants in cells (~2-fold over-expression; OE). It should also be noted that throughout the manuscript, we use commercially available Locked Nucleic Acids (LNAs) for RNAi, which demonstrate improved stability and functionality (Elmen, J *et al*. (2005) *Nucleic Acids Research*).

4. siRNA western blots are in the supplemental but not referred to in the text. Five supplemental movies are included, but they are also not referred to in the text. Obviously if the data is important enough to include in the manuscript, it should be referred to.

We apologize for this mistake, we have now referenced these data within the revised manuscript.

5. The first paragraph comes across as a collection of observations that the authors then assume will make sense to the reader and they can follow the logic. In fact, it is not at all clear how these observations support the preliminary conclusions stated by the authors.

Assuming that the reviewer is referring to the first paragraph in the results section, it was our intention to describe a set of observations that motivated the rest of the work.

6. The difference between control and Lamin A OE in figure 1D are quite small. It would be helpful to include representative images either in the main figure or as a supplemental.

We agree with the reviewer, the difference in nuclear aspect ratio for cells over-expressing (OE) EGFP alone and Lamin A are quite small. As other metrics (e.g., leader bleb area) were more substantially affected, we did not draw conclusions from changes in nuclear aspect ratio.

7. I question the utility of the conceptual figures (1K, 2J, and 4M). I don't think the data presented fully supports these conceptual relationships and it comes across as wishful thinking or potentially misleading. This type of conceptualization should be restricted to the discussion.

The conceptualizations are intended to help readers to understand the sometimes non-intuitive relationships revealed by our data. For instance, that the up- and down-regulation of emerin is found to decrease migration through increasing nuclear stiffness.

8. Should incorporate lack of adhesion into the hypothesized pathways (e.g., figure 1B)? How would integrin-based adhesion change the outcome of these pathways? What is the significance of the nucleus being in the leader bleb?

Thank you for this suggestion, we have now incorporated lack of adhesion into the pathway presented in figure 4A. We speculate that in cells with focal adhesions, that the nucleus may be subjected to increased pulling forces. By increasing the Src mediated phosphorylation of emerin, the nucleus may be stiffer in cells using mesenchymal migration. Although it is not entirely clear what the significance is, cells tend to migrate faster when the nucleus is positioned in leader blebs (Logue, JS *et al.* (2018) *Nature Protocol Exchange*).

9. Why would emerin siRNA lead to rounder nuclei? Should they not be more easily deformed?

Using cells treated with Lat-A (i.e., removing cortical actin), cells were found to be stiffer after emerin RNAi (figure 3D). As these data suggest an increase in nuclear stiffness, emerin RNAi led to rounder nuclei.

10. For figure 3, it is really not clear the logic of treating with LA, or why nuclear F-actin is mentioned. It heavily implies that nuclear stiffness is not measured in these cells. I question then, why is the data included as nuclear stiffness?

Using our assay, stiffness is primarily regulated by cortical contractility. Therefore, in order to measure the stiffness of intracellular organelles, we removed F-actin using Lat-A. However, as emerin has been shown to bind the pointed end, we wondered if emerin may require F-actin for nuclear stiffening. Therefore, we turned to isolated nuclei for measuring stiffnesses. Indeed, this revealed that emerin requires F-actin for nuclear stiffening.

11. I am not convinced based on the imaging data presented that emerin delta INM is not still in the nuclear envelope. Can this shift be quantified somehow?

We apologize for the confusion, this mutation shifts the localization of emerin away from the inner to the Outer Nuclear Membrane (ONM), which was quantified in the original publication (Pfaff, J *et al.* (2016) *Journal of Cell Science*). Accordingly, emerin ΔINM is enriched at the ONM and contiguous ER (figure S2B; arrows). In agreement with this data, the over-expression (OE) of emerin ΔINM does not increase the stiffness of nuclei (figure 3E-F).

12. Major question: Does this cell type actually have the Lamin A/C nuclear stiffening mechanism?

Consistent with Lamin A being a core component of the nucleoskeleton, the over-expression (OE) of Lamin A is shown to increase the stiffness of nuclei (figure 3E-F).

13. Is figure 4I different data then that presented in figure 3G? If it is the same, that should be explicitly stated in the paper.

For comparison to emerin (Y74F/Y95F), we reproduced data for EGFP alone and emerin WT from the previous figure. This has now been stated in the legend for figure 4 within the revised manuscript.

14. The FRET experiments are also missing key controls as well as indications of how equivalent levels of reporter expression were imaged in the different experiments. At a minimum, they should show that treating cells expressing the reporter with a Src inhibitor has the expected effect on the FRET signal.

Although cells with roughly equivalent levels of fluorescence were imaged, emission ratios (acceptor/donor) and FRET efficiencies effectively normalize the data to biosensor levels. Consistent with the original publication, emission ratios (acceptor/donor) were lowered in freshly plated (spherical) cells by treatment with the Src family kinase inhibitor, Dasatinib (0.5 μM; 10 min) (figure 5A) (Ouyang, M *et al*. (2008) *PNAS*).

Reviewer 2

Comments for the Author:

The work by Lavenus et al. adds new important mechanistic data supporting the model in which the nucleus non-transcriptionally converts mechanical cues into chemical signals impacting cell morphodynamics in mechanically restrictive microenvironments. The authors present a series of experimental manipulations and quantitative analyses to illuminate a rather nonintuitive feature of biophysical processes involved in amoeboid cell motility and cell shape control, which is nonlinearity. Indeed, based on their experiments, Lavenus et al. show that interfering with the ability of the nuclear envelope to be stretched in response to cellular compression affects blebbing cell morphodynamics. Specifically, the authors show that emerin-dependent modulation of nuclear stiffness plays a decisive role: nuclear stiffness that is either too small or too high is detrimental to stretching of the nuclear envelope, suggesting that an optimal nuclear envelope stiffness should be maintained in order for the nucleus to be stretchable. Lavenus et al. propose that emerin-dependent control of nuclear envelope stretchability can affect the activity of the nuclear stretch-sensitive protein cPLA2, which in turn impacts the signaling to the actomyosin cortex and blebbing cell morphodynamics. I am curious whether the authors can provide any experimental evidence in support of this proposal. One can think of a simple experiment in which the low contractility/blebbing phenotype downstream of emerin-dependent modulation of nuclear stiffness (and thus cPLA2 recruitment to nuclear/perinuclear ER membranes) can be rescued by the addition of (1) ionomycin to artificially boost levels of intracellular Ca2+ allosterically activating cPLA2 and/or (2) purified arachidonic acid – the product of cPLA2's enzymatic activity. I believe these experiments are relatively easy to perform within a reasonable time frame and their results may add to the completeness of the story, therefore reinforcing its conclusions.

To bolster our claim, we measured rates of transmigration through 8 μm pores for non-targeting, emerin, cPLA2, and emerin + cPLA2 RNAi cells. In emerin and cPLA2 RNAi cells, transmigration was reduced to a similar degree (figure 2K). Importantly, we did not observe an additive effect on transmigration in emerin + cPLA2 RNAi cells, which suggests they are in the same pathway (figure 2K & 4A).

For reasons that are unclear at this time, we are unable to increase blebbing with ionomycin, arachidonic acid, or ionomycin + arachidonic acid.

Within the revised manuscript, we also now show that emerin RNAi reduces transmigration through 8 pores, whereas transmigration through 12 μm pores is unaffected (figure 2J). Thus, the requirement for emerin is specific to cells migrating through mechanically constrictive microenvironments.

In response to reviewer 1, the proposed pathway has been de-emphasized. Instead, alternative mechanisms are now discussed.

Reviewer 3

Advance Summary and Potential Significance to Field:

In this interesting manuscript, authors show that Emerin regulates leader bleb formation in cells migrating under confinement. This study adds to the breadth of knowledge accumulating with regards to nuclear dynamics in blebbing cells migrating under confinement.

Reviewer 3

Comments for the Author:

1. Authors suggest that levels of these proteins affect migration. The data suggests that migration speed of all cells is reduced because a smaller percentage of cells now adopt the leader mobile (LM) phenotype. Migration of LM cells themselves is not altered (Fig. 1H, Fig. 2G, and Fig. 4G). As such, Lamin A/Emerin are upstream regulators of bleb dynamics, but I am not sure I would conclude they impair migration per se. Maybe some simple rephrasing would be required.

Thank you for pointing this out, we have now rephrased the manuscript to describe the observed phenotypes more accurately.

2. On the other hand, as part of their mechanism, authors argue that there are changes in actomyosin contractility driving migration, but all they quantify are changes in leader bleb formation. To prove actomyosin contractility is involved I would like to see some quantification of MLC2 phosphorylation levels and/or careful analysis of pMLC2 and actin localization. In supplemental figure 4, F-actin levels are measured by FACS but more careful assessment of localization is required.

Within the revised manuscript, we have now included representative images of actin and myosin in cells over-expressing (OE) EGFP alone, emerin WT, and Y74F/Y95F (supplemental figure 5). Despite our best efforts, we were unable to quantify a difference in the level of myosin at the cell periphery.

To bolster our claim, we measured rates of transmigration through 8 μm pores for non-targeting, emerin, cPLA2, and emerin + cPLA2 RNAi cells. In emerin and cPLA2 RNAi cells, transmigration was reduced to a similar degree (figure 2K). Importantly, we did not observe an additive effect on transmigration in emerin + cPLA2 RNAi cells, which suggests they are in the same pathway (figure 2K & 4A).

Within the revised manuscript, we also now show that emerin RNAi reduces transmigration through 8 pores, whereas transmigration through 12 μm pores is unaffected (figure 2J). Thus, the requirement for emerin is specific to cells migrating through mechanically constrictive microenvironments.

In response to reviewer 1, the proposed pathway has been de-emphasized. Instead, alternative mechanisms are now discussed.

3. Levels of Lamin A and Emerin after knockdown and over-expression should be shown by WB and/or ideally at single cell level. I think this is required to validate their proposed mechanisms. An attempt was made in Fig. 1J and shows quite a spread of over-expression. Authors could over-express different levels of Lamin A and Emerin to show it is a concentration dependent effect. In those lines, are all the emerin constructs expressed to the same level? A Western blot would help.

Thank you for pointing this out, Western blots of emerin and Lamin A/C levels after RNAi are shown in supplemental figure 1A. For Lamin A (Fig. 1H), emerin WT (Fig. 2H), ΔINM (Fig. S2I), Q133H (Fig. S3H), and Y74F/Y95F (Fig. 4L), we used Immunofluorescence (IF) to conduct a cell-by-cell analysis of fold over-expression (OE). Transfected cells were identified using the fluorescent protein tag. Lamin A was found to be over-expressed by ~1.5-fold, whereas emerin WT and mutants were found to be over-expressed by ~2-fold. For imaging, the very low and high expressing cells (i.e., outliers) were excluded; therefore, the average values from these data

for fold over-expression are representative of the actual level of each protein.

4. Some quantification of cell viability is also required. Manipulating the nuclear envelope can have detrimental effects on cells so it would be good to confirm how 'healthy' these cells are.

We agree, data for viability - cells that are alive after 5 hr of fluorescence imaging – are now provided in supplemental figure 6A-B. For RNAi and over-expression (OE) cells, there were no statistically significant differences in viability.

5. The graphs with % cells (Fig. 1E, G, Fig. 2D, F, and Fig. 4D, F) do not have any stats or error bars on them. These should be provided.

As these represent pooled (categorical) data, we have now used a chi-squared (x^2) test wherever possible. To reinforce our conclusions, additional statistical tests are used in related continuous data.

6. Are all the experiments carried out with the 3 micron confinement? This could be reiterated in some of the figure legends for clarity. If authors are using 3 micron confinement for most of the work, Transwell assays in Fig. 1 should also use pores of that size rather than 8 microns.

Thank you for pointing this out, we have made sure that all the figures reiterate that cells are confined down to ~3 µm.

As the deformability of the nucleus can in general regulate migration through confined environments, we have used filters with differing pore sizes as a general approach for measuring rates of motility. Transmigration assays have been used in this way previously, such as in Lomakin, A *et al*. (2020) *Science*. Additionally, we have found that very few melanoma cells are able to migrate through 3 µm pores.

7. Authors have only used a cell line, A375-M2 metastatic melanoma line, with very high levels of intrinsic actomyosin. Could they repeat key experiments to see what happens with a cell line with low actomyosin levels to start with, does it form less LM?

When confined down to ~3 µm, we have found that transformed cells will use fast amoeboid (leader bleb-based) migration at similar rates. In contrast, we have observed that primary cells, such as Human Foreskin Fibroblasts (HFFs), using mesenchymal migration. We speculate that in cells with focal adhesions, that the nucleus may be subjected to increased pulling forces. By increasing the Src mediated phosphorylation of emerin, the nucleus may be stiffer in cells using mesenchymal migration. This idea may be tested in a separate study.

Second decision letter

MS ID#: JOCES/2021/259493

MS TITLE: Emerin regulation of nuclear stiffness is required for fast amoeboid migration in confined environments

AUTHORS: Sandrine B Lavenus, Karl W Vosatka, Alexa P Caruso, Maria F Ullo, Ayesha Khan, and Jeremy S Logue
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.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, two reviewers gave favourable reports. Reviewer 1, however, does not recommend publication and says that the overall conclusions of your study are confusing. They do suggest that a major re-write might help clarify the message and having re-read your MS I do have to agree that a few tweaks might help. Before I accept your paper, I would appreciate if you could revise you text in light of reviewer 1's comments to help bring out your message as this will help our readership.

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

In their revised manuscript, Lavenus et al seek to understand how emerin activity governs leader bleb-based migration of confined A375-m2 melanoma cells.
Despite their revisions, the overall conclusions of the manuscript remain confusing and perhaps poorly supported by the data presented. The authors conclude that fast amoeboid migration is inhibited by nuclear stiffness, but they don't consistently say what aspect is inhibited. For example, in figure 1 it seems clear that lamin A expression increases the number of cells using classic amoeboid cancer cell movement (No Leader; NL) and does not reduce the speed of cells that use leader bleb-based movement. In other words, lamin A expression prevents cells from switching to leader bleb-based movement, it does not inhibit fast amoeboid migration directly. The same holds true for emerin knockdown and overexpression, more cells use classic amoeboid cancer cell migration and those cells that still use leader blebs move at the same speed. If the major migration phenotype as a result of perturbing lamin/emerin/src is less cells switching to leader bleb-based movement, it is not clear why this pathway or nuclear stiffness would promote cells to use leader bleb-based movement. I think the exact research question that is being addressed by this manuscript is unclear and this lack of clarity is propagated through the results and discussion as well. For example, in referring to figure 1I the authors state
"Thus, increasing levels of Lamin A correlate with increased nuclear stiffness and a reduction in motility". But they do not measure nuclear stiffness and do not clearly define what they mean by reduction in motility and how that is related to the leader bleb-based migration. This lack of precision when describing how their results related to leader bleb-based migration is found throughout the manuscript and is a major factor in my difficulty in understanding the significance of the work presented.

Comments for the author

Overall, this manuscript requires a major re-write and potentially additional data. As it stands, the overall conclusions of the paper are not clear and/or not well supported by the data presented.

Reviewer 2

Advance summary and potential significance to field

I think the authors have done a good job in addressing the majority of concerns expressed by all the reviewers. I, therefore, recommend the manuscript for publication.

Just one note, Reviewer #3 mentioned: "6. Are all the experiments carried out with the 3 micron confinement? This could be reiterated in some of the figure legends for clarity. If authors are using 3 micron confinement for most of the work, Transwell assays in Fig. 1 should also use pores of that size rather than 8 microns."

From the physical and geometric point of view, a round opening with the diameter 3 um is NOT equal to parallel confinement, where the distance between the two confining surfaces is 3 um. I hope we all can agree on that.

Comments for the author

I think the authors have done a good job in addressing the majority of concerns expressed by all the reviewers. I, therefore, recommend the manuscript for publication.
Just one note, Reviewer #3 mentioned: "6. Are all the experiments carried out with the 3 micron confinement? This could be reiterated in some of the figure legends for clarity. If authors are using 3 micron confinement for most of the work, Transwell assays in Fig. 1 should also use pores of that size rather than 8 microns."
From the physical and geometric point of view, a round opening with the diameter 3 um is NOT equal to parallel confinement, where the distance between the two confining surfaces is 3 um. I hope we all can agree on that.

Reviewer 3

Advance summary and potential significance to field

authors have answered all my concerns and I consider this work is now ready for publication

Comments for the author

N/A

Second revision

Author response to reviewers' comments

Author responses are in red

Reviewer 1

Advance Summary and Potential Significance to Field:

In their revised manuscript, Lavenus *et al.* seek to understand how emerin activity governs leader bleb-based migration of confined A375-M2 melanoma cells. Despite their revisions, the overall conclusions of the manuscript remain confusing and perhaps poorly supported by the data presented. The authors conclude that fast amoeboid migration is inhibited by nuclear stiffness, but they don't consistently say what aspect is inhibited. For example, in figure 1 it seems clear that Lamin A expression increases the number of cells using classic amoeboid cancer cell movement (No Leader; NL) and does not reduce the speed of cells that use leader bleb-based movement. We have now added, 'no leader (NL) cells are also non-mobile' to the manuscript text

for added clarity. There is no reference to “classic amoeboid cancer cell movement” in the manuscript text. In other words, Lamin A expression prevents cells from switching to leader bleb-based movement, it does not inhibit fast amoeboid migration directly. The same holds true for emerin knockdown and overexpression, more cells use classic amoeboid cancer cell migration and those cells that still use leader blebs move at the same speed. Correct, increased nuclear stiffness prevents the transition to fast amoeboid (leader bleb-based) migration. This has been stated in the manuscript text, for example, on page 6, first paragraph, we state: Therefore, the transition to the leader mobile (LM) phenotype was inhibited by Lamin A OE. If the major migration phenotype as a result of perturbing Lamin/emerin/Src is less cells switching to leader bleb-based movement, it is not clear why this pathway or nuclear stiffness would promote cells to use leader bleb-based movement. Our data are consistent with a model involving the nuclear membrane stretch sensor, cPLA2, which promotes actomyosin contractility in response to nuclear deformation (Lomakin, A *et al*. (2020) *Science* and Venturini, V *et al*. (2020) *Science*).
See figures 1J, 2J-K, and 4A. I think the exact research question that is being addressed by this manuscript is unclear and this lack of clarity is propagated through the results and discussion as well. In the last two paragraphs of the Introduction, we discuss the potential significance of emerin/Src in regulating nuclear membrane stretch, cPLA2, and the transition to fast amoeboid (leader bleb-based) migration. For example, in referring to figure 1I the authors state “Thus, increasing levels of Lamin A correlate with increased nuclear stiffness and a reduction in motility”. But they do not measure nuclear stiffness and do not clearly define what they mean by reduction in motility and how that is related to the leader bleb-based migration. Using isolated nuclei, nuclear stiffnesses were measured in figures 3F-G and 4I-J. The relationship between nuclear stiffness, cPLA2, and the transition to fast amoeboid (leader bleb-based) is discussed throughout the manuscript text. This lack of precision when describing how their results related to leader bleb-based migration is found throughout the manuscript and is a major factor in my difficulty in understanding the significance of the work presented.

Reviewer 1

Comments for the Author:

Overall, this manuscript requires a major re-write and potentially additional data. As it stands, the overall conclusions of the paper are not clear and/or not well supported by the data presented.

Please see our point-by-point responses above.

Third decision letter

MS ID#: JOCES/2021/259493

MS TITLE: Emerin regulation of nuclear stiffness is required for fast amoeboid migration in confined environments

AUTHORS: Sandrine B Lavenus, Karl W Vosatka, Alexa P Caruso, Maria F Ullo, Ayesha Khan, and Jeremy S Logue
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.