Vimentin tunes cell migration on collagen by controlling β1 integrin activation and clustering

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MS TITLE: Vimentin tunes cell migration by acting as an adaptor that enables the assembly of the molecular complex that controls β1 integrin functions

AUTHORS: Zofia Ostrowska-Podhorodecka, Isabel Ding, Wilson Lee, Jelena Tanic, Sevil Abbasi, Pamma D. Arora, Richard S. Liu, Alison E. Patteson, Paul A. Janmey, and Christopher A. McCulloch
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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, both reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns with major revisions, clarifications as well as strengthening of data and conclusions. 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

This manuscript presents a substantial amount of new information concerning the role of vimentin in the assembly of cell-substrate adhesion complexes as well as in integrin activation and adhesive function involving collagen. The topic addressed is interesting in attempting to delineate the structural and functional relationships between vimentin and integrins. The findings presented are potentially interesting, and they are strengthened by the use of both vimentin knockdown and knockout approaches using two different mouse cell lines. Although this paper could potentially provide a useful contribution, its presentation and some of the results are extremely difficult to interpret without some additional experimental information and most importantly, clearer and more precise presentation of the conclusions.
At present, this potentially useful manuscript would require major revisions for further consideration.

Comments for the author

Although the authors could be commended for attempting to draw clear conclusions from their series of potentially quite interesting but currently rather incomplete and confusing experiments and interpretations, some conclusions do not seem correct or are incomplete. This reviewer would strongly encourage the authors to provide a series of essential clarifications, crucial additional experimental information, and revisions.

1. The title is too imprecise and potentially misleading. It states that vimentin “enables the assembly of the molecular complex that controls beta1 integrin functions.” The actual experimental results presented in this paper do not in my opinion support the very strong claims of this title. First of all, the authors do not demonstrate that vimentin “enables” assembly, but it instead modulates integrin adhesion size. The second conclusion that this molecular complex controls integrin functions is in my opinion a strong over-statement, since it only modulates or affects activation and function of one class of integrins. Finally, the conclusions of this paper may only apply to collagen as a ligand.

2. The concept that vimentin “controls” activation of the beta 1 integrin would be potentially interesting, but the authors fail to acknowledge that this particular integrin also plays central roles in cell adhesion to fibronectin as well as collagen. One critically important set of experiments that is missing involves whether the effects observed are due to adhesion to monomeric collagen and would not be observed on fibronectin. That is, does lack of vimentin have any effect on integrin activation and binding of fibronectin-coated beads if the cells are cultured on fibronectin? If the effects are seen solely on collagen, the title, abstract, text, and discussion should make this point clear.
It would also be important for the authors to check the wording of their conclusions throughout the text, which in some cases appear to be over-statements. For example, the claim is made on page 7 “We conclude that vimentin filaments are required for the linkage of beta 1 integrin activation with cluster formation.” This reviewer does not feel that the analyses are strong enough to make this strong claim.

3. The title, abstract, and text is not written sufficiently clearly to clarify the role of vimentin. Specifically, it is important to compare the specific observations with the conclusions that can be drawn: The authors show interesting findings that turn out to be somewhat counter-intuitive when one states the findings compared to the apparent conclusions as follows. Results: Loss of vimentin

results in increased beta1 activation reduced clustering of this integrin, and increased adhesion strength to collagen along with inhibition of paxillin recruitment with reduced Cdc42 activation and migration. Interpreting these findings to ask the role of vimentin, we see that vimentin seems to be involved in reducing integrin activation, increasing integrin clustering in focal adhesions, and reducing adhesion strength to collagen! Of course, with enhancing paxillin and Cdc42 activation. These important and puzzling interpretations are not presented or discussed by the authors to explain how the normal levels of vimentin actually suppress integrin activation and adhesion to collagen. This confusing disconnect needs to be discussed, since an alternative view is that vimentin serves to negatively regulate integrin functions.

4. The interpretation of these findings becomes particularly confusing when we examine what the authors report concerning the binding of fibrinogen and fibronectin beads. In this case, the loss of vimentin results in a decrease in binding. When we turn that around to ask what does this say about the role of vimentin, it seems to indicate that vimentin can activate integrins involved in not only fibrinogen but also fibronectin binding, the latter of which is presumably uses the same beta1 integrin as for collagen. Instead of the current relatively simple interpretations of the data by the authors, a potentially even more interesting interpretation is that vimentin plays roles in both inhibition and activation of integrin functions depending on the context (collagen compared to fibronectin). Again, a critical missing piece of information is whether lack of vimentin shows the reported effects on integrin activation and function on a fibronectin-coated substrate.

Otherwise, various statements in the text that are too general will need to be modified, e.g., “vimentin filaments are likely involved in the formation and maturation of focal adhesions” and “a key role of vimentin filaments in the regulation of focal adhesions.” Other statements besides these in the conclusion will also need to be revised if the effects are only seen on monomeric collagen and not on fibronectin or other surfaces.

5. A central element of this study involves the role of vimentin in cell adhesion complexes, at least to collagen. There appears to be a contradiction in the data presented. The text states that talin is increased by more than 7-fold in what appear to be adhesion complexes, yet the blotting of collagen adhesion-associated proteins is stated to show that “vimentin depletion did not markedly alter the abundance of talin or beta1 integrin.” The data shown in Figure 4 A shows a very slight increase in talin along with the loss of paxillin that suddenly becomes the focus of the authors. It needs to be clarified whether there is some change in the amount of talin associated with adhesions, and especially how the authors feel that vimentin actually directly “controls beta1 integrin function.”

Less crucial concerns:

6. The authors compare the behavior of cells on surfaces coated with collagen compared to fibronectin, but they never indicate how much of each protein was used to coat the surface, nor whether there were equal amounts that were actually present on the surface. A simple alternative approach to confirm their findings would be to examine for effects of vimentin depletion on different coating concentrations of these two proteins.

A more minor concern is that the authors examine cells adhering to monomeric collagen that was likely pepsin-cleaved to remove all the normal non-triple-helical collagen regions if purchased from Advanced BioMatrix, whereas the collagen found in vivo is fibrillar and full-length. One wonders whether the morphological effects would be the same if cells were plated on more physiological fibrillar collagen. Fortunately, the experiments using beads apparently did not use fibrillar collagen, but all of those on collagen did.

7. The authors should be commended for their extent of quantification in numerous graphs. What is never clarified, however, is how often the western blots were repeated, and whether the same results were obtained, especially concerning the rather puzzling findings concerning talin and paxillin in collagen cell adhesion complexes.

8. The mass spectrometry of collagen-associated adhesion complexes is potentially valuable, but there are some concerns as follows. First, it is not clear why the vimentin knockout shows the presence of considerable spectral counts for vimentin itself. In fact, the number of counts for the

vimentin scaffold spectrum count of 133 is substantially higher than the counts for talin and alpha-actinin, which are indicated as being increased.
Also, since this is the key evidence for talin enrichment, how often was the mass spec analysis performed?

Minor points:

9.The beads assay uses 2 micron beads and is performed over three hours. It seems likely that there will be considerable bead endocytosis during this period. Could the effects also involve a major complement of increased endocytosis? The mass spectrometry results show an increase in clathrin heavy chain at adhesions, which could be significant.

10. The claim at the bottom of page 8 following the demonstration that talin deletion reduced integrin activity more in null compared to control cells
“indicating that vimentin does indeed affect beta1 integrin activity” could be suggested but is definitely not proven by the data.

11. Figure 7 looks like a direct feed-forward loop that should self-reinforce. Does inhibiting Cdc42 mimic all the morphological and focal adhesion effects of vimentin knockout?

12. Figure 7 also shows the beta1 integrins binding to each other, but they actually bind to alpha subunits. Also, cytoskeleton is misspelled in this diagram.

13. Can the authors rule out the possibility that the deletion of vimentin causes more direct effects on Rho GTPase activities? Other signaling systems including other Rho GTPases were not examined.

14. In the discussion starting on page 13, the authors suddenly start referring to increases in plasma membrane-associated beta 1 integrin at least twice, but the only relevant results seem to be the flow cytometry for activated beta1 integrin. If they did not perform membrane fractionation experiments to support this claim concerning this integrin, the authors need to use more precise wording.

15. Other points:
a. On page 16, what is meant by 10% antibiotics?
b. On page 19, the Cdc42 activation assay was presumably performed by blotting with anti-Cdc42 antibody.
c. On page 20, the elution of proteins from collagen-coated beads was stated to involve 10 mM DTT at 60 degrees for 1h. Is this correct?
d. Possible typo: under Acknowledgements, the authors referred to “the Jiji macro” but do they mean “Fiji”?

Reviewer 2

Advance summary and potential significance to field

Vimentin is a well established EMT relate protein, yet its functional relevance in EMT and the motility of mesenchymal cells is still not fully understood. In the submitted paper the authors have investigated the possibility that vimentin would function in cell migration through regulating b1-integrin adhesions and via recruiting paxillin into focal complexes, enabling Cdc42-dependent PAK1 activation and thus supporting cell extension formation on collagen. This is a potentially interesting study but needs some revisions to be more convincing.

Comments for the author

Specific points.
The vimentin silencing experiments lack specifity control for off-target effects. Please include more than 1 independent siRNA or a rescue.
Figure 2A. Please include the blot for vimentin to show silencing efficacy in this specific experiment.

Figure 2C. Please provide representative images of the 9EG7 staining that was analysed. The rather dramatic b1-integrin activation (3-10 fold) must look striking and would be lovely to include in the paper.
Figure 3A. It is not obvious, in these images, that the 9EG7 staining intensity would be substantially higher in the vim lacking cells. Why? Investigating the integrin clusters could be done with a total (non-activation specific) b1-antibody. Figure 3B. How were the clusters defined? Analysed?
Figure 4A. It would be important to show the protein inputs alongside the collagen bead pulldowns.
Figure 4G. Talin silencing lacks the necessary gold-standard off-target controls mentioned above. For the effect on integrin activation, only relative effects of the silencing on activity are shown. The authors conclude in the text "Loss of talin reduced integrin activity by 50 % in mEF null cells and by 25 % in control cells" does this mean that in the talin silenced mEF the integrin activity remains still significantly higher than in the control cells (if the integrin activity is 10-fold higher in the null mEF?). Please again include representative cell images.
Figure 5. Pax silencing lacks the necessary gold-standard off-target controls mentioned above. What is the effect of pax silencing on the integrin clusters/cell morphology/protrusions Figure 6C-E. It would be good to include the vim null cells here as controls to show that the effect is specific to PAK mediated phosphorylation of vimentin. 6J. Why are the Bg intensities of the pictures so different? What is the effect of the PAKi on b1-integrin activity in the vim lacking cells?

Minor.
Figure 2D, y-axis has a typo Figure 2E-H is it bounded or bound?
Figure 6A, please indicate the boarders of the spliced gels clearly

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First revision

Author response to reviewers' comments

Michael Way, Ph.D.
Editor
Journal of Cell Science

Dear Dr. Way,
Manuscript No.: JOCES/2020/254359
Title: Vimentin tunes cell migration on collagen by controlling β1 integrin activation and clusterin g
Thank you for arranging for the review of this manuscript. We have made substantial modifications to the manuscript that are directly in response to the very helpful comments of the reviewers. We have addressed the reviewers' comments with a large number of new experiments and by extensive re-writing of the text. Below we provide a point-by-point response to each of the reviewers' comments that are in italicized text; our responses are in plain text.

REVIEWERS' COMMENTS
Reviewer #1:
1. *The title is too imprecise and potentially misleading. It states that vimentin "enables the assembly of the molecular complex that controls beta1 integrin functions." The actual experimental results presented in this paper do not in my opinion support the very strong claims of this title. First of all, the authors do not demonstrate that vimentin "enables" assembly, but it instead modulates integrin adhesion size. The second conclusion that this molecular complex controls integrin functions is in my opinion a strong over-statement, since it only modulates or affects activation and function of one class of integrins. Finally, the conclusions of this paper may only apply to collagen as a ligand.*
We thank the Reviewer for this suggestion, and we agree that the proposed title was imprecise and potentially misleading. We have re-written the title as: '**Vimentin tunes cell migration on collagen by controlling ß1 integrin activation and clustering**', which in our opinion better reflects our main findings. The data presented in this manuscript strongly support the claim that vimentin plays a crucial role in modulation of ß1 integrin function relating to collagen adhesion, especially with

respect to activity and avidity, which in turn regulates cell migration through collagen matrices.

2. *The concept that vimentin "controls" activation of the beta 1 integrin would be potentially interesting, but the authors fail to acknowledge that this particular integrin also plays central roles in cell adhesion to fibronectin as well as collagen. One critically important set of experiments that is missing involves whether the effects observed are due to adhesion to monomeric collagen and would not be observed on fibronectin. That is, does lack of vimentin have any effect on integrin activation and binding of fibronectin-coated beads if the cells are cultured on fibronectin?*
We thank the Reviewer for this suggestion and have added new ß1 integrin activation and cell migration data (Fig. S1 A-B), which show that loss of vimentin did not impact ß1 integrin activation when cells were plated on a fibronectin (FN)-coated surface (Fig. S1 A) or were attached to FN-coated fluorescent beads (Fig. S1 B). We agree with the reviewer that these experiments would support the statement that the effects observed are due to adhesion to monomeric Fig. 2D and fibrillar collagen Fig. 2C but would not be observed on fibronectin.

The Reviewer asked if '*lack of vimentin has any effect on integrin activation and binding of fibronectin-coated beads if the cells are cultured on fibronectin'*.
This proposed "double coating experiment" was conducted (i.e., incubation of FN-coated beads with cells cultured on FN-coated surfaces; Fig. S1 D and G) and these data showed that the addition of FN-coated beads to WT and null mEFs that were cultured on FN-coated surfaces resulted in a 2-fold increase of bead binding by vimentin null mEFs (Fig. S1 D). Further, analysis of the relative strength of binding of FN-coated beads to cells plated on fibronectin, showed slightly stronger bead binding by vimentin null mEFs compared with wild type cells (Fig. S1 G). These results suggest that increasing ligand accessibility enhanced ß1 integrin activity relating to fibronectin adhesion in vimentin null mEFs.

If the effects are seen solely on collagen, the title, abstract, text, and discussion should make this point clear. It would also be important for the authors to check the wording of their conclusions throughout, results and discussion to reflect this point. *the text, which in some cases appear to be over-statements. For example, the claim is made on page 7 "We conclude that vimentin filaments are required for the linkage of beta 1 integrin activation with cluster formation." This reviewer does not feel that the analyses are strong enough to make this strong claim.*
We thank the Reviewer for these insights and accordingly we edited the text of the title, abstract and discussion. We have been careful to delineate the restriction of the meaning of binding to collagen and fibronectin throughout the text. We also modified the text in the manuscript according to the reviewer's suggestions and have been careful to not over-state our conclusions as the reviewer has correctly pointed out. Now the statement highlighted by the reviewer has been re-written as follows: "We conclude that vimentin filaments are involved in linking collagen- dependent ß1 integrin activation with cluster formation." (page 8). To support this statement that vimentin-dependent regulation of ß1 integrin activation is strictly dependent on binding to collagen matrices, we have re-written the relevant paragraph in the Discussion (p. 17, middle).

3. *The title, abstract, and text is not written sufficiently clearly to clarify the role of vimentin. Specifically, it is important to compare the specific observations with the conclusions that can be drawn: The authors show interesting findings that turn out to be somewhat counter-intuitive when one states the findings compared to the apparent conclusions as follows. Results: Loss of vimentin results in increased beta1 activation, reduced clustering of this integrin, and increased adhesion strength to collagen along with inhibition of paxillin recruitment with reduced Cdc42 activation and migration. Interpreting these findings to ask the role of vimentin, we see that vimentin seems to be involved in reducing integrin activation, increasing integrin clustering in focal adhesions, and reducing adhesion strength to collagen! Of course, with enhancing paxillin and Cdc42 activation. These important and puzzling interpretations are not presented or discussed by the authors to explain how the normal levels of vimentin actually suppress integrin activation and adhesion to collagen. This confusing disconnect needs to be discussed, since an alternative view is that vimentin serves to negatively regulate integrin functions.*
We appreciate the Reviewer's in-depth suggestions. To clarify the role of vimentin in the proposed processes we modified the text as requested and highlighted these very modifications throughout the Results and Discussion. The Reviewer's main point is that "*These important and puzzling interpretations are not presented or discussed by the authors to explain how the normal levels of vimentin actually suppress integrin activation and adhesion to collagen".* To address this issue, we

have re-written the text as follows on page 17: “The formation of integrin clusters is thought to depend on interacting processes that regulate the production, concentration and diffusion of integrin-activating proteins (Welf et al., 2012). Our findings indicate that vimentin affects the organization, structure, and function of cell-collagen adhesions by suppressing β1 integrin activation, thereby resulting in well-organized, mature integrin clusters. In contrast, loss of vimentin dissipates local concentrations of integrins, which curtails the growth of integrin clusters and collagen adhesions.” Along the same lines, we also made substantial changes to the title (as suggested in comment number 1 above) and in the Abstract (page 2).

4. *The interpretation of these findings becomes particularly confusing when we examine what the authors report concerning the binding of fibrinogen and fibronectin beads. In this case, the loss of vimentin results in a decrease in binding. When we turn that around to ask what does this say about the role of vimentin, it seems to indicate that vimentin can activate integrins involved in not only fibrinogen but also fibronectin binding, the latter of which is presumably uses the same beta1 integrin as for collagen. Instead of the current relatively simple interpretations of the data by the authors, a potentially even more interesting interpretation is that vimentin plays roles in both inhibition and activation of integrin functions depending on the context (collagen compared to fibronectin). Again, a critical missing piece of information is whether lack of vimentin shows the reported effects on integrin activation and function on a fibronectin-coated substrate.*
We thank the Reviewer for this important point. In the modified manuscript we clarify our previous, somewhat non-specific explanation that relates to this point. Vimentin’s opposing roles in adhesion, inhibition and activation of integrins for different substrates (collagen versus fibronectin and fibrinogen) suggested to us that other adhesion receptors are involved in regulating focal adhesion formation, perhaps through the involvement of co-dominant integrins that interact with certain adaptor proteins in the adhesions. Thus in contrast to cell adhesion to collagen, in which only the β1 integrin sub-unit is involved, fibronectin is a ligand for the β1, β3, and β7 integrins. Cognizant of the complexity which the Reviewer has correctly pointed out, we added new data on cell adhesion to fibronectin, and modified the text in the manuscript (pages 6-7) that relates to integrin activation and function. This text describes cell behavior on fibronectin-coated substrates (shown in Fig. S1 A-C, E, F). These data show that there is no difference of integrin activation and binding to fibronectin between vimentin WT and Vim null mEFs, when fibronectin coating concentrations on beads or dishes are ~10 μg/ml. When we increased the overall abundance of fibronectin-coated substrates (i.e., on beads and dishes), there was increased β1 integrin activation in vimentin null mEFs compared with WT cells (Fig. S1 D and G). These data indicated that vimentin plays a role in regulating integrin activation in a manner that depends on β1 integrin accessibility to matrix ligand. Note that these data apply to fibronectin. In the context of adhesion to collagen, we consider that the β1 integrin is central to recruiting vimentin to adhesions. In contrast, for fibronectin, although the β1 integrin is one of the integrins used by cells for adhesion to fibronectin, other abundantly-expressed fibronectin-binding integrins (e.g. β3 and β7) may exhibit difference in their cytoplasmic domains that preclude their interactions with vimentin in the same manner that is the focus of this manuscript.

According to the literature, fibrinogen binds to cells through β2 and β3 integrins (Hantgan et al., 2010) but not through β1 integrins. With this background, fibrinogen was used as a negative control for vimentin-dependent β1 integrin regulation.

Another part of this Reviewer’s comment was, *“Otherwise, various statements in the text that are too general will need to be modified, e.g., “vimentin filaments are likely involved in the formation and maturation of focal adhesions” and “a key role of vimentin filaments in the regulation of focal adhesions.”*
We thank the Reviewer for these insights. We modified the text in the manuscript, which now is written as: “Our findings indicate that vimentin affects the organization, structure, and function of cell-collagen adhesions by suppressing β1 integrin activation, thereby resulting in well- organized, mature integrin clusters. In contrast, loss of vimentin dissipates local concentrations of integrins, which curtails the growth of integrin clusters and collagen adhesions.”

The reviewer also points out that: “*Other statements besides these in the conclusion will also need to be revised if the effects are only seen on monomeric collagen and not on fibronectin or other surfaces.*
We modified the title of point three in the Results paragraph, which is “Vimentin affects the

clustering and spatial distribution of the ß1 integrin on collagen".

5. *A central element of this study involves the role of vimentin in cell adhesion complexes, at least to collagen. There appears to be a contradiction in the data presented. The text states that talin is increased by more than 7-fold in what appear to be adhesion complexes, yet the blotting of collagen adhesion-associated proteins is stated to show that "vimentin depletion did not markedly alter the abundance of talin or beta1 integrin." The data shown in Figure 4 A shows a very slight increase in talin along with the loss of paxillin that suddenly becomes the focus of the authors. It needs to be clarified whether there is some change in the amount of talin associated with adhesions, and especially how the authors feel that vimentin actually directly "controls beta1 integrin function."*

We thank the Reviewer for these insights and agree that the original western blots were not in agreement with the mass spectrometry data and that the explanation was confusing. Accordingly, we pursued this issue experimentally in detail. We conducted new mass spectrometry analyses: these results are presented in Fig. S3 A. We also repeated the western blot analyses of the collagen bead-associated proteins. Initial assessments of collagen bead-associated proteins (presented as the ratio of protein abundance for mEF vimentin null vs. mEF vimentin WT cells) showed a ~2.5-fold increase in the abundance of talin in the adhesions after vimentin depletion. We replaced the original blot with a new one (Fig. 4A), which shows essential increase of talin in the collagen-beads associated proteins. We included an additional panel that shows the whole cell protein inputs, which indicate that loss of vimentin did not affect whole cell talin expression.

Analysis of whole cell lysates by immunoblotting indicated that the total abundance of ß1 integrin in vimentin null mEFs was somewhat higher than vimentin WT mEFs. However, analysis of the collagen bead-associated proteins showed equal amounts of ß1 integrin in WT and null cells, suggesting that when equivalent concentrations of collagen are presented on bead surfaces, they bind an equivalent amount of ß1 integrin receptors. Collectively these data indicate that the observed increase of ß1 integrin activation in vimentin null cells is related to the higher abundance of talin levels in the adhesions and talin-dependent, inside-out integrin signaling. We modified the text in the manuscript (p. 8, 9 and 16) to reflect these new findings and interpretations.

Less crucial concerns:

6. *The authors compare the behavior of cells on surfaces coated with collagen compared with fibronectin, but they never indicate how much of each protein was used to coat the surface, nor whether there were equal amounts that were actually present on the surface. A simple alternative approach to confirm their findings would be to examine for effects of vimentin depletion on different coating concentrations of these two proteins. A more minor concern is that the authors examine cells adhering to monomeric collagen that was likely pepsin-cleaved to remove all the normal non-triple-helical collagen regions if purchased from Advanced BioMatrix, whereas the collagen found in vivo is fibrillar and full-length. One wonders whether the morphological effects would be the same if cells were plated on more physiological fibrillar collagen. Fortunately, the experiments using beads apparently did not use fibrillar collagen, but all of those on collagen did.*

We thank the Reviewer for these insights and helpful suggestions. We agree that the annotated information was missing in the manuscript and should be clarified. We analyzed both fibronectin and collagen using more quantitative, ligand dose-response experiments (see Fig. A below). The flow cytometry data showed that the optimal bead coating concentrations for fibronectin and collagen (based on similar levels of bead recruitment) was 10 mg/ml and 1 mg/ml, respectively (see Fig. B below). While monomeric collagen was used at 0.1 mg/ml concentration for coating, the integrin activation results obtained with this coating corresponded to those experiments that were obtained with beads coated with fibrillar collagen at 1 mg/ml. The information about collagen and fibronectin concentrations is now provided in more detail in the text on pages 5 and 6.

We agree with the Reviewer's view that fibrillar collagen provides a more physiologically relevant substrate than monomeric collagen. Accordingly, we plated WT and null cells on fibrillar collagen gels (1 mg/ml) for 4, 8, and 16 h (see Fig. C below [NOTE: We have removed a figure which was provided for the reviewers in confidence]) to assess whether the levels of vimentin expression affect cell morphology. Confocal imaging showed similar morphologies for vimentin WT and null cells when plated on monomeric or fibrillar collagen. In this context, the Reviewer wondered about what form of collagen was used in experiments. Whereas experiments that examined cell morphology, WB analysis, and ß1 integrin activation (detected by KMI6 or 9EG7 ß1 integrin antibodies) were

performed with monomeric collagen, bead binding and bead recruitment analyses were conducted with fibrillar collagen. We found that the results from these assays that were conducted with fibrillar or monomeric collagen, under the conditions used here, were not different. As cell detachment (needed for flow cytometry) is more reproducible with monomeric collagen, and to maintain consistency between experiments, we frequently used monomeric collagen for these assays.

7. *The authors should be commended for their extent of quantification in numerous graphs. What is never clarified, however, is how often the western blots were repeated, and whether the same results were obtained, especially concerning the rather puzzling findings concerning talin and paxillin in collagen cell adhesion complexes.*
We thank the Reviewer for this query. All western blot experiments were done at least three times on different days and with separate cultures. This information and related experimental details are now included in each figure legend. We have also included in the Fig. S3 B-E, histograms that show 'protein of interest'/ß-actin ratio. These data were quantified based on western blots prepared from whole-cell lysates. The size of the error bars (standard deviations) provides information on the variation of results that are presented.

8. *The mass spectrometry of collagen-associated adhesion complexes is potentially valuable, but there are some concerns as follows. First, it is not clear why the vimentin knockout shows the presence of considerable spectral counts for vimentin itself. In fact, the number of counts for the vimentin scaffold spectrum count of 133 is substantially higher than the counts for talin and alpha-actinin, which are indicated as being increased. Also, since this is the key evidence for talin enrichment, how often was the mass spec analysis performed?*
We appreciate the Reviewer's suggestions and note that vimentin peptides were indeed detected in cells nominally described as Vim null cells. We obtained new, earlier passage vimentin null mEFs and analysed 3 separate groups of cultures (i.e. three separate mass spectrometry analyses). The new data are presented as protein abundance ratios for vimentin WT versus null mEF (Fig. S3 A). Quantification of these abundance ratios showed that the ratio of vimentin in null vs. WT is 0.03 ($p<0.001$), which indicates that vimentin peptides are present at very low levels. This result is supported by new vimentin immunoblotting data shown in Fig. 4A.

Minor points:

9. *The bead assays use 2 micron beads and are performed over three hours. It seems likely that there will be considerable bead endocytosis during this period. Could the effects also involve a major complement of increased endocytosis? The mass spectrometry results show an increase in clathrin heavy chain at adhesions, which could be significant.*
We thank the Reviewer for these insights. In the Materials and Methods section of the previous version of the manuscript (page 18), the bead time incubation was indicated as 3 hours. The correct information is that bead-binding assays were performed after two hours of incubation (page 19, Materials and methods and in the figure legends). We corrected this error in the manuscript and in the description for Fig. 2D, where this information was missing. Notably, our previous data (Bozavikov et al., 2014) showed that 1 µm beads were partially internalized by Rat- 2 fibroblasts over 3 h of incubation in the presence of serum. To minimize endocytosis for the current experiments, we utilized larger (2 µm) beads which were incubated with cells in serum- free medium, two approaches that greatly reduce bead internalization. This information is now included in the modified manuscript (page 20).
In the context of the Reviewer's concern about the increase in clathrin heavy chain observed in the bead-associated proteins and the potential relationship of this finding to endocytosis, our earlier data (Bozavikov et al., 2014) showed collagen and fibronectin-coated bead internalization used actin-dependent and not clathrin-dependent processes.

10. *The claim at the bottom of page 8 following the demonstration that talin deletion reduced integrin activity more in null compared to control cells "indicating that vimentin does indeed affect beta1 integrin activity" could be suggested but is definitely not proven by the data.*
We thank the Reviewer for these insights. We corrected this statement (p.8) which now is written as 'These data support the notion that vimentin plays a role in regulating integrin function activation or inhibition depending on the access of the ß1 integrin to collagen or fibronectin, respectively.'

11. *Figure 7 looks like a direct feed-forward loop that should self- reinforce. Does inhibiting Cdc42 mimic all the morphological and focal adhesion effects of vimentin knockout?*
We thank the Reviewer for these suggestions. We analyzed the effect of Cdc42 inhibition on cell morphology and focal adhesions. Vimentin WT and null mEFs were incubated with ML141 (10 μM), a Cdc42 inhibitor. Confocal images (Fig. S4 C) and data shown in Fig. S3 indicate that inhibition of Cdc42 does not mimic all of the morphological (Fig. S4 E) and focal adhesion (Fig. S4 F-I) effects of vimentin depletion when cells are plated on fibronectin-coated surfaces. In contrast, various measures of cell morphology (Fig. S4 D, E) and focal adhesions (Fig. S4 F-I) show that ML141 treatment of WT cells phenocopies vimentin null mEFs that were plated on collagen. These analyses support our view that vimentin controls cell behavior in a collagen- dependent manner. We rewrote the text (page 11) to reflect these ideas.

12. *Figure 7 also shows the beta1 integrins binding to each other, but they actually cooperate with alpha subunits to mediate matrix adhesion. Also, cytoskeleton is misspelled in this diagram.*
We thank the Reviewer for this suggestion and corrected the errors in this figure.

13. *Can the authors rule out the possibility that the deletion of vimentin causes more direct effects on Rho GTPase activities? Other signaling systems including other Rho GTPases were not examined.*
We appreciate the Reviewer's suggestion but cannot exclude that deletion of vimentin causes more direct effects on Rho GTPase activity. We agree that loss of vimentin may impact other proteins that are members of the Rho GTPase family. Accordingly, we performed a new pulldown assay experiment to analyze Rac1 activity to address this insightful question. The data show that loss of vimentin has a direct effect on Rho GTPase activity. Loss of vimentin was associated with a 94% reduction of Rac1 activity, which was restored by 57% after vimentin re- expression (Fig. 5B). We have inserted these new data into the text (page 10).

14. *In the discussion starting on page 13, the authors suddenly start referring to increases in plasma membrane-associated beta 1 integrin at least twice, but the only relevant results seem to be the flow cytometry for activated beta1 integrin. If they did not perform membrane fractionation experiments to support this claim concerning this integrin, the authors need to use more precise wording.*
We appreciate the intent of this comment but note that the total abundance of plasma membrane-associated β1 integrin was analysed and these data are presented in Fig. 2B. In this experiment we used an antibody against the β1 integrin (clone KMI6) to measure integrin abundance on the surface of non-permeabilized cells. This information is now included in Results and is considered in the Discussion and figure legend. We have re-written the paragraph on page 6 in the Results section to provide a more satisfactory description.

15. **Other points:**

a. On page 16, what is meant by 10% antibiotics?
We now indicate that cells were cultured in medium contained 10% (v/v) antibiotics.

b. On page 19, the Cdc42 activation assay was presumably performed by blotting with anti-Cdc42 antibody.
We thank the Reviewer for this suggestion and have added the information that samples were immunoblotted with a Cdc42 antibody (Abcam; Cambridge, MA).

c. On page 20, the elution of proteins from collagen-coated beads was stated to involve 10 mM DTT at 60 degrees for 1h. Is this correct?
This information is correct. We obtained the protocol description from the Hospital for Sick Children SPARC BioCentre (Toronto, ON, Canada), where the protein preparation and mass spectrometry analysis were performed.

d. Possible typo: under Acknowledgements, authors referred to "the Jiji macro" but do they mean "Fiji"?

This modification has been done.

Reviewer 2 Comments for the Author:

1. The vimentin silencing experiments lack specificity control for off-target effects. Please include more than 1 independent siRNA or a rescue.
In accordance with the Reviewer's suggestion, we have included in Fig. 3 A-C and Fig. 5A new data showing vimentin rescue experiments. Vimentin re-expression in mEF null cells (Fig. S2 A) restored β1 integrin cluster size by 42% compared with mEF WT cells (Fig. 3B). Contemporaneously, after vimentin rescue, there were only 11% more clusters compared with control, whereas loss of vimentin resulted in >55% increase of small (~0.5 μm) β1 integrin clusters (Fig. 3C).

2. Figure 2A. Please include the blot for vimentin to show silencing efficacy in this specific experiment.
This new immunoblot is now shown in Fig. 2A.

3. Figure 2C. Please provide representative images of the 9EG7 staining that was analysed. The rather dramatic b1-integrin activation (3-10 fold) must look striking and would be lovely to include in the paper.
We thank the Reviewer for the suggestion. We included a new figure (Fig. 2E) with images of the 9EG7 staining, which are similar to the results shown in the earlier Fig. 2C (currently Fig. 2D). All images in Fig. 2E were obtained with the same parameters of laser power and master gain using a Zeiss LSM800 confocal microscope system. During the image conversion using the Fiji software, we adjusted the minimal and maximal gray value to 3 and 192, respectively, and we used these settings for all images that were analyzed.

4. Figure 3A. It is not obvious in these images that the 9EG7 staining intensity would be substantially higher in the vim lacking cells. Why? Investigating the integrin clusters could be done with a total (non-activation specific) b1-antibody. Figure 3B. How were the clusters defined? Analysed?
We thank the Reviewer for these suggestions and agree that the provision of 9EG7 staining intensity would be expected to be substantially higher in the Vim null cells. At this juncture we wanted to present β1 integrin cluster size and distribution independent of fluorescence intensity. Accordingly, we measured the fluorescence intensity (Fig. 3A) in such a manner that we obtained a similar fluorescence signal that enabled demonstration of the morphological differences of the integrin clusters. To address the very reasonable question of β1 integrin fluorescence intensity, we added a new panel in the Fig. 2E that shows fluorescence intensity of β1 integrin for all analyzed cells.
According to the literature (Welf et al., 2012), ligand binding to the β1 integrin extracellular domain promotes conformational changes that increase ligand affinity, modify protein-interaction sites in the cytoplasmic domains, which then lead to the resulting signaling. In addition to conformational changes, multivalent ligand binding leads to integrin clustering. We defined 'integrin clusters' as small, localized regions where high concentrations of bound integrins are concentrated. These clusters exhibited higher fluorescence intensity compared with background and were analyzed by Fiji software that used the macro described in the section in the Materials and Methods that is entitled- *Immunostaining and microscopy analysis*.
We agree that investigating the integrin clusters could be done with a total (non-activation specific) β1-antibody as we had used an antibody (9EG7) that recognizes an activation epitope in the β1 integrin chain. We extended these analyses of β1 integrin clusters using a rat anti-mouse β1 integrin (KMI6, Abcam Cat. # ab95623) that is an integrin activation independent antibody. The results obtained from confocal imaging of cells plated on FN or Col-coated surfaces for 3 h (Fig. S2 D-F) showed considerable similarities to those data on integrin activation and clustering that were obtained with the 9EG7 antibody. We modified the text on page 7-8 to reflect this information.

5. Figure 4A. It would be important to show the protein inputs alongside the collagen bead pulldowns.
We thank the Reviewer for the suggestion and these data are now included in Fig. 4A.

6. Figure 4G. Talin silencing lacks the necessary gold-standard off-target controls mentioned above. For the effect on integrin activation, only relative effects of the silencing on activity are shown. The authors conclude in the text "Loss of talin reduced integrin activity by 50 % in mEF null cells and by 25 % in control cells" does this mean that in the talin silenced mEF the integrin activity remains still significantly higher than in the control cells (if the integrin activity is 10-fold higher

in the null mEF?). Please include representative cell images.
We agree on the need for gold-standard, off-target controls for talin KD. We replaced the previous experimental data with new analyses. We investigated whether talin is involved in how vimentin regulates cell-collagen interactions. We studied the binding of collagen-coated 2 µm beads in vimentin WT and null cells after talin deletion and re-expression (Fig. 4G, Fig. S2 F). Our findings showed that after 2 h incubation, loss of talin reduced bead recruitment by 68% in mEF WT and vimentin null cells. Analysis of talin rescue cells showed that talin re-expression resulted in 55% and almost complete restoration of collagen-coated bead binding for mEF WT and Vim null cells compared with their respective controls (Fig. 4H).

Mice Talin1 3'UTR CGC and CAG were obtained from Horizon (Lafayette, CO).
1. Ms Tln1 siRNA 3'UTR CGC: Custom siRNA, Standard 0.015 µmol Regular; length 21, molecular weight 13270.1g/mol, 10nmol, 132.7mg

CTM-639351
MCCCQ-000001

Sense Sequence as ordered:
CGCUCCAAGAGUAUUAUUAdTdT
Antisense Sequence as ordered:
UAAUAAUACUCUUGGAGCGdTdT

2. Ms Tln1 siRNA 3'UTR CAG: Custom siRNA, Standard 0.015µmol Regular; length 21, molecular weight 13285.1g/mol, 10nmol, 132.8mg.
CTM-639352
MCCCQ-000003

Sense Sequence as Ordered:
CAGGUCAGACUCCAAUAAAdTdT
Antisense Sequence as Ordered:
UUUAUUGGAGUCUGACCUGdTdT
The oligonucleotide has been converted to the 2'-hydroxyl, annealed, and desalted duplex.

Figure 5. Pax silencing lacks the necessary gold-standard off-target controls mentioned above. What is the effect of pax silencing on the integrin clusters/cell morphology/protrusions.
We thank the Reviewer for these insights and suggestions. We added paxillin rescue data (Fig. 5C) and have re-written the text on page 10 to reflect these new data. The paxillin KD and rescue efficiency are presented in Fig. S5 A.
Analysis of the paxillin silencing effect on the cell morphology was assessed based on cell size and the number of cell extensions (Fig. S5 D and C, respectively). The data showed no substantial differences for cells plated on fibronectin or collagen. In contrast, measurements of the ß1 integrin cluster size (Fig. S5 H) showed a marked decrease in ß1 integrin cluster size after paxillin KD in cells cultured on fibronectin and collagen. Quantification of the number of ß1 integrin clusters (Fig. S5 I) indicated the additive effect of vimentin and paxillin silencing when cells were cultured on collagen-coated surfaces.

Figure 6C-E. It would be good to include the vim null cells here as controls to show that the effect is specific to PAK-mediated phosphorylation of vimentin. 6J. Why are the Bg intensities of the pictures so different? What is the effect of the PAK1 on ß1-integrin activity in the vim lacking cells?
We thank the Reviewer for this insight. As indicated on pages 10 and 29, cells were stained for vimentin and all imaging and quantification in Fig. 6C-E focussed on vimentin filaments. As these images were intended to report on the effect of PAK on the structure of vimentin filaments, it is not possible to conduct these experiments using vimentin mEFs vim null cells as these cells do not exhibit vimentin filaments.
Based on the Reviewer's comments on the variation of the image background, we think that apparent differences in background may have arisen during the PDF compression that was part of the conversion to a smaller file size that is needed for review purposes. We have improved the resolution of the new Fig. 6J as suggested by the Reviewer.
Further, we analyzed the effect of the PAK1 mutants on the ß1 integrin activity in the WT and Vim null mEFs. The histogram (Fig. S6 C) shows that inhibition of PAK1 kinase (K299R) caused a slight

(15%) reduction of ß1 integrin activity in vimentin null cells and a more substantial reduction (38%) for mEF WT cells.

Minor.
-Figure 2D, y-axis has a typo
The correction of the y-axis in Fig. 2D was done as suggested.

-Figure 2E-H is it bounded or bound?
We corrected this error in Fig. 2E-H.

-Figure 6A, please indicate the borders of the spliced gels clearly
In accordance with the Reviewer's suggestion, we have included borders for the spliced gels more clearly in Fig. 6A.

Second decision letter

MS ID#: JOCES/2020/254359

MS TITLE: Vimentin tunes cell migration on collagen by controlling β1 integrin activation and clustering

AUTHORS: Zofia Ostrowska-Podhorodecka, Isabel Ding, Wilson Lee, Jelena Tanic, Sevil Abbasi, Pamma D. Arora, Richard S. Liu, Alison E. Patteson, Paul A. Janmey, and Christopher A. McCulloch
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 gave favourable reports but reviewer 1 still raised some 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 manuscript presents a substantial amount of new information concerning the role of vimentin in the assembly of cell-substrate adhesion complexes as well as in integrin activation and adhesive function involving collagen. The topic addressed is interesting in attempting to delineate the structural and functional relationships between vimentin and integrins. The findings presented are potentially interesting, and they are strengthened by the use of both vimentin knockdown and knockout approaches using two different mouse cell lines. Although this paper could potentially provide a useful contribution, it would require further revision.

Comments for the author

In this revised manuscript, the authors have provided substantial amounts of new data, revised conclusions, and clarifications that have greatly strengthened this study to the extent that it may now ultimately become acceptable for publication after appropriate revisions that are much less major than originally. Although this reviewer remains dissatisfied by the explanations of how vimentin may affect integrin activation and clustering the findings themselves appear interesting and seem reasonable to consider publishing even without a clear mechanistic interpretation of how they function physiologically. For example, the Discussion states “the association of vimentin with paxillin and talin enables the maturation of integrin clusters and the maintenance of collagen adhesion structures that are involved in inside-out integrin signaling” yet the actual data clearly show that loss of vimentin actually increases adhesion to collagen and integrin activation. Although the findings do not fit nicely into current concepts of integrin function in which activation enhances clustering and function, this reviewer supports reporting experimental findings and leaving it to the field to explain them.

1. The authors refer often to the property of integrin clustering or clusters, yet the data remain somewhat confusing. Specifically, even though the quantification in Figure 3B appears convincing, the quantitative loss of integrin cluster size in 3T3 cells is just not visible in terms of the areas of integrin clusters. Specifically, it would be valuable for the authors to show the quantitative data for the specific images shown, since the clusters in panel A for 3T3 cells and mEF for Control compared to VIM siRNA or VIM null just do not seem to agree with their quantifications of surface area of clusters.

2. The authors refer to integrin clustering or clusters, which in the literature generally refers to its initial appearance in aggregates. In this paper, the clusters or aggregates are substantial in size, and the text becomes quite confusing when referring to focal complexes compared to focal adhesions. This reviewer suggests that the authors clarify their findings with respect to relatively standard nomenclature in the integrin field where focal complexes or nascent adhesions are tiny, transient structures that are precursors to larger, more stable focal adhesions. Importantly, to members of the integrin field, it would be very helpful for the authors to clarify whether their findings refer to a role for vimentin in the size of focal adhesions, which would be the interpretation of this reviewer after carefully examining the fluorescence images, rather than the conversion of focal adhesions to focal complexes after it is depleted.

3. The revised summary Figure 7 is improved, but it now clearly shows vimentin binding directly to the cytoplasmic tails of both the alpha and beta subunits of integrins. Yet the abstract states: “We propose that vimentin tunes cell migration through collagen by acting as an adaptor protein for focal adhesion proteins” and the authors cite publications indicating that its interaction with a collagen-binding integrin is indirect. For readers who turn to diagrams to understand the conclusions of a paper, it would be much better for the authors to indicate schematically much more clearly that vimentin is somehow interacting indirectly with integrins by functioning as an adapter protein for other proteins in cell adhesions rather than serving as a direct binding partner

Minor points:

4. Are the results in Figure 5E not statistically significant?

5. Although the effects are not as major as those involving integrin interactions with collagen, there does seem to be evidence for effects on cell interactions with fibrinogen and occasionally fibronectin. For completeness, the authors should ideally mention this fact in the Discussion.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all my concerns with new experimentation, inclusion of key controls and clarification of their experimental design. The strong b1-integrin activation upon loss of vimentin is very interesting and would be great to see in the future, if the authors are able to define the underlying mechanism in even greater detail.

Comments for the author

no further revision needed

Second revision

Author response to reviewers' comments

Michael Way, Ph.D.
Editor, Journal of Cell Science

Dear Dr. Way,
Manuscript No.: JOCES/2020/254359
Title: **Vimentin tunes cell migration on collagen by controlling ß1 integrin activation and clustering**
Thank you for arranging for the review of this manuscript. We have made modifications to the manuscript that are directly in response to the very helpful comments of Reviewer 1. We have addressed point-by-point these comments that are in italicized text; our responses are in plain text.

REVIEWER #1 COMMENTS
1. *The authors refer often to the property of integrin clustering or clusters, yet the data remain somewhat confusing. Specifically, even though the quantification in Figure 3B appears convincing, the quantitative loss of integrin cluster size in 3T3 cells is just not visible in terms of the areas of integrin clusters. Specifically, it would be valuable for the authors to show the quantitative data for the specific images shown, since the clusters in panel A for 3T3 cells and mEF for Control compared to VIM siRNA or VIM null just do not seem to agree with their quantifications of surface area of clusters.*
We thank the Reviewer for this insight about cluster size quantification. To address this issue, we modified Fig. 3A by inserting as overlays on the images, examples of ß1 integrin clusters that are marked circumferentially with a red line. The clusters that are outlined are those integrins that stain above threshold intensity levels that were used during the analyses of the ß1 integrin cluster. The number (per cell) and area of the ß1 integrin clusters were obtained using the Analyze Particles procedure in Fiji. Due to the large number of integrin clusters per cell, each data point shown in the histograms in Fig. 3B represents an average value for all of the ß1 clusters in a single cell. This observation may explain potential differences between certain specific clusters that may be seen in the images shown here and that are of a broad size distribution, and data from the large number of analyzed cells in the data shown in Fig. 3B. To facilitate visual assessment of the ß1 integrin clusters in mEF control cells we replaced the previously shown mEF control image with a new one. We also re-wrote the legend for Fig. 3A (p. 29) to reflect the reviewer's point.

2. *The authors refer to integrin clustering or clusters, which in the literature generally refers to its initial appearance in aggregates. In this paper, the clusters or aggregates are substantial in size,*

and the text becomes quite confusing when referring to focal complexes compared to focal adhesions. This reviewer suggests that the authors clarify their findings with respect to relatively standard nomenclature in the integrin field, where focal complexes or nascent adhesions are tiny, transient structures that are precursors to larger, more stable focal adhesions. Importantly, to members of the integrin field, it would be very helpful for the authors to clarify whether their findings refer to a role for vimentin in the size of focal adhesions, which would be the interpretation of this reviewer after carefully examining the fluorescence images, rather than the conversion of focal adhesions to focal complexes after it is depleted.
We thank the Reviewer for these insights and the suggestion. We agree that the conventional definition of "focal complexes" should be restricted to newly formed, small, transient structures that are in most instances, precursors to larger, more stable focal adhesions. The term "focal adhesion" generally refers to more stable and larger adhesion structures that form mechanical links between actin filaments and the underlying matrix. According to the Reviewer's comment we modified the text where indicated (pages 4, 7, 9, 12, 13, and 16).

3. *The revised summary Figure 7 is improved, but it now clearly shows vimentin binding directly to the cytoplasmic tails of both the alpha and beta subunits of integrins. Yet the abstract states: "We propose that vimentin tunes cell migration through collagen by acting as an adaptor protein for focal adhesion proteins" and the authors cite publications indicating that its interaction with a collagen-binding integrin is indirect. For readers who turn to diagrams to understand the conclusions of a paper, it would be much better for the authors to indicate schematically much more clearly that vimentin is somehow interacting indirectly with integrins by functioning as an adapter protein for other proteins in cell adhesions rather than serving as a direct binding partner.*
We thank the Reviewer for this comment. Based on the cited literature and our understanding of the biology, vimentin filaments co-localize with the ▫2▫1 integrin. Co- localization suggests a spatial relationship between two entities (in this case, proteins) but the term does not indicate that the entities physically interact. Indeed, the proposed use of the word "indirect" may not necessarily clarify the development of the notion that we are attempting to develop in this part of the paper. In order to show in the diagram that the interaction between vimentin and integrins is indeed indirect, we inserted a red arrow between vimentin filaments and integrins to underline their specific spatial relationship while **NOT** suggesting that they have a direct physical interaction. This approach was also used to indicate that other pairs of proteins, based on the literature, co-localize and contribute to the formation of focal adhesions. We have also modified the description of Fig. 7 in the legend (p. 32).

Minor points:
Are the results in Figure 5E not statistically significant?

We thank the Reviewer for this observation. We did insert the asterisks in the figure, which indicate statistically significant differences for mEF WT cells treated with ML141 where necessary. As there are a large number of points and lines shown in the histogram, asterisks are inserted above the control sample (mEF WT). No significant changes were found in the data demonstrating PAK phosphorylation in mEF null cells. This note has been added to the main text in the manuscript (p. 10).

Although the effects are not as major as those involving integrin interactions with collagen, there does seem to be evidence for effects on cell interactions with fibrinogen and occasionally fibronectin. For completeness, the authors should ideally mention this fact in the Discussion.

We thank the Reviewer for this suggestion. We agree that the Discussion is focused on collagen and does not address some of our observation that relate to other matrix proteins. Due to the Journal of Cell Science word limit for manuscripts, we were not able to expand the Discussion further in the context of cell interactions with fibrinogen or fibronectin, which do not have a significant impact on vimentin-dependent ▫1 integrin activation. Nevertheless, we did indicate that fibronectin exerted a limited impact on cell morphology (bottom of page 13). According to the Reviewer's suggestion we provided some additional information about mEF interactions with fibrinogen and fibronectin in the Discussion for completeness (page 14).

Third decision letter

MS ID#: JOCES/2020/254359

MS TITLE: Vimentin tunes cell migration on collagen by controlling β1 integrin activation and clustering

AUTHORS: Zofia Ostrowska-Podhorodecka, Isabel Ding, Wilson Lee, Jelena Tanic, Sevil Abbasi, Pamma D. Arora, Richard S. Liu, Alison E. Patteson, Paul A. Janmey, and Christopher A. McCulloch
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.