JCB

Hemidesmosomes modulate force generation via focal adhesions

Wei Wang, Alba Zuidema, Lisa Molder, Leila Nahidiazar, Liesbeth Hoekman, Thomas Schmidt, Stefano Coppola, and Arnoud Sonnenberg

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May 22, 2019

Re: JCB manuscript #201904137

Prof. Arnoud Sonnenberg
The Netherlands Cancer Inst.
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Plesmanlaan 121
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Dear Arnoud,

Thank you for submitting your manuscript entitled "Role of hemidesmosomes in cellular force regulation". Your manuscript has now been evaluated by three reviewers. While the three reviewers find the study overall well-done and the crosstalk between hemi-desmosomes and focal adhesions very interesting, they have brought up significant conceptual and mechanistic concerns about the suitability of the advance for JCB. Rev #1 and #3 both appear to question the physiological relevance of their findings, while both Rev#2 and #3 indicate the level of molecular mechanism is currently insufficient for JCB. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

Given that successfully addressing all reviewer concerns seems both a substantial and open-ended concern, we are concerned there is a high chance that the manuscript may be rejected after re-review. Therefore, we could consider an open-door rejection allowing you to outline how you will address the reviewer concerns regarding the physiological relevance of your study and/or the molecular mechanism underlying the crosstalk between FAs and HD. For instance, additional results on the role of plectin or intermediate filaments in the control of RhoA and YAP activity or indications of a direct interaction between FAs and HD would certainly improve the manuscript. We would be happy to run your proposition by the reviewers to see if your proposed revisions stand a chance at success before you decide to undertake any major additional work. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission. However, If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

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

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

Sincerely,

Sandrine Etienne-Manneville, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

The interplay of extracellular matrix adhesions in epidermal cells remains enigmatic. It is generally believed that hemidesmosomal adhesions together with their attached keratin intermediate filaments serve a more static function whereas focal adhesions and their associated acto-myosin stress fibers support cell shape changes and movement. The authors investigate the still unresolved crosstalk between both junctions in epidermal keratinocytes.

They find that immortalized keratinocytes lacking hemidesmosomal a6b4 integrin, with disrupted hemidesmosomal keratin linkage, compromised keratin F-actin interaction or hemidesmosomal binding to the extracellular matrix ligand laminin-332 all induce enlarged focal adhesions resulting in increased cell spreading and increased traction forces. This is accompanied by aVb5 integrin redistribution from clathrin lattices to focal adhesions. Furthermore, the authors provide evidence that hemidesmosomes suppress tension-generating signaling pathways as well as nuclear YAP accumulation. From this they conclude that hemidesmosomes suppress tensile forces of FA-anchored acto-myosin fibers.

Overall, the conclusions are well supported by the experimental data and provide novel insights. A major caveat, however, is that the observations are limited to static cultured cells. The authors should provide evidence that the observed phenomena relate to a physiologically relevant situation.

The authors base their observations on the assumption that hemidesmosomes are lost in wounded and migrating keratinocytes. This view has been challenged by in vivo observations (Underwood et al., 2009, J Histochem Cytochem 57:123) and observations in cultured cells (e.g., Tsuruta et al., 2003, Cell Motil Cytoskeleton 54:122; Tsuruta et al., 2011, J Dermatol Sci 62:1; Pora et al., 2019, J Invest Dermatol doi: 10.1016/j.jid.2019.03.1139. [Epub ahead of print]). How can the published observations be reconciled with the data presented in the manuscript?

Additional issues:
Figures 1A/B, 2B: The merged images in the mutant cells insinuate nuclear b4 integrin staining. Please indicate in the pictures that it is due to DAPI staining as specified in the figure legends.

Figure 4B: In contrast to the labeling I can only detect green (actin) and blue (DAPI). Please, improve.

Figure 4C: The legend claims that an intensity profile is shown for actin, which, however, I cannot find. The legend also erroneously refers to B and not to the left image in C.

Figure 5B: n should be provided.

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

This manuscript addresses the interplay of focal adhesions (FA) and hemidesmosomes (HD). It suggests that HD, possibly via mechanical coupling of keratins to the FA-anchored actin cytoskeleton, control cell spreading and actomyosin-dependent traction forces.
Overall, the data are of high quality. They raise the question how HD control FA and traction forces. The authors find reduced RhoA activity and increased nuclear YAP/TAZ in the absence of b4 integrin but do not address the molecular mechanism by which these changes take place and how they link to the HD control of FA. There are some issues that require experiments to she light on the underlying molecular mechanisms:
1. The decrease om active RhoA in PA-JEB/b4 keratinocytes is rather modest compared to the high decrease in P-MLC, raising the question whether additional mechanisms are involved. This should be investigated. Further, it remains to be shown that changes in b4 affect RhoA activity. It may be helpful to use RhoA biosensors.
2. In the absence of b4 and in mutant cell lines, YAP is translocated to the nucleus. This is an interesting finding, however, no data are provided to investigate the corresponding mechanism and to link it to the overall phenotype. This should be experimentally addressed.
3. Understanding how HD mediate the crosstalk with FA to control traction forces is key to the overall story. Plectin is implied to play a significant role in this but no molecular evidence is provided. This should be done.
4. The following papers should be discussed:
Correia et al., J Cell Biol. 1999 Aug 23;146(4):831-42
Seltmann et al., J Invest Dermatol. 2013 Jan;133(1):181-90. doi: 10.1038/jid.2012.256
Hiroyasu et al., FASEB J. 2016 Jun;30(6):2298-310. doi: 10.1096/fj.201500160R
Pora et al., J Invest Dermatol. 2019 Apr 2. pii: S0022-202X(19)31446-0. doi: 10.1016/j.jid.2019.03.1139

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

The manuscript by Wang et al. investigates the role of hemidesmosomes in modulating force generation via focal adhesions. Overall, the study presents some new and interesting findings on the cross-talk between these two adhesive structures and insight into epithelial mechano-sensing. The experiments are well designed, and the data are of good quality. However, there are a couple important mechanistic details that should be examined in more depth in order to support the authors proposed model. Specific comments are listed below

1. There does not appear to be clear evidence of a physical linkage between HDs and FAs as indicated in the discussion and Fig 7. While this can be inferred by the known roles of plectin and the mechanical data presented here, it is an important unanswered question of exactly how and where the two are linked? Perhaps proximity ligation assays or IPs would be one way to address this question?
2. Why does the R1281W mutant not fully rescue cell spreading and force generation on the micropillar? Could this possibly indicate that plectin binding does not completely mediate force coupling between FAs and HDs? Perhaps analysis of PlecKO cells in this assay would be insightful?
3. The authors describe the role of HDs in modulating FA maturation, but only FA size is quantified. It would be good to include measurements of FA length and staining for additional FA components, such as paxillin or zyxin.
4. Another key question is what are the effects of altered mechano-transduction via HDs on cell phenotype? Are there proliferative or migratory defects as might be affected by changes in FA

maturation and YAP localisation.
5. In the methods section it would be helpful to provide more detail on cell line generation and clonality rather than referring to previous publications? Similarly in the figure captions, a clearer description of the vector only or transfection controls would be helpful.
6. The line scan data is Fig 3 does not add much as it depends on how the lines are drawn and only represents cells in one image. The same qualitative information can be observed from the IF images alone, and the quantitative measurement of fluorescence across many cells in 3C is more convincing.
7. In Fig 4, it would be good to show a super-resolution image of the beta4(-) cells as well to see how association between Ifs and FAs compares with beta4(+) cells.

Reviewer #1

1. Comment: A major caveat, however, is that the observations are limited to static cultured cells. The authors should provide evidence that the observed phenomena relate to a physiologically relevant situation. The authors base their observations on the assumption that hemidesmosomes are lost in wounded and migrating keratinocytes. This view has been challenged by in vivo observations (Underwood et al., 2009, J Histochem Cytochem 57:123) and observations in cultured cells (e.g., Tsuruta et al., 2003, Cell Motil Cytoskeleton 54:122; Tsuruta et al., 2011, J Dermatol Sci 62:1; Pora et al., 2019, J Invest Dermatol doi: 10.1016/j.jid.2019.03.1139. [Epub ahead of print]). How can the published observations be reconciled with the data presented in the manuscript?

Response: We thank the reviewer for these useful comments and agree that we should provide more evidence for the physiological relevance of our study. To fully reconcile the published observations with our data and to address the issue of the physiological relevance, we have presented *in vitro* wound healing assays in Fig. 1A. In these assays, we show that type I hemidesmosomes are disassembled and cellular tension and assembly of mature focal adhesions are increased in the cells at the wound interface. These findings confirm our results indicating that keratinocytes can only counterbalance cellular tension if HDs are fully assembled and connected to the IF network through plectin.

Integrin β4 and other HD components could still be observed in some cells at the wound edge, yet their expression and organization into type I HDs was very limited. Therefore, these cells might not be able to efficiently counteract cellular tension. We discuss our observations and those described in the suggested literature in more detail in the discussion (line 143-145 and 415-432).

2. Comment: Additional issues about Figure legends.

Figures 1A/B, 2B: The merged images in the mutant cells insinuate nuclear b4 integrin staining. Please indicate in the pictures that it is due to DAPI staining as specified in the figure legends.

Figure 4B: In contrast to the labeling I can only detect green (actin) and blue (DAPI). Please, improve.

Figure 4C: The legend claims that an intensity profile is shown for actin, which, however, I cannot find. The legend also erroneously refers to B and not to the left image in C.

Figure 5B: n should be provided.

Response: We have revised the figures, corrected the legends and provide the missing information.

Reviewer #2

1. Comment: The decrease om active RhoA in PA-JEB/β4 keratinocytes is rather modest compared to the high decrease in p-MLC, raising the question whether additional mechanisms are involved. This should be investigated. Further, it remains to be shown that changes in β4 affect RhoA activity. It may be helpful to use RhoA biosensors.

Response: To provide evidence that the increased actomyosin generated tension in the β4-deficient cells are mediated through a RhoA-controlled pathway, we treated the β4-deficient cells and plectin-deficient with the ROCK inhibitor (Y-27632), and the myosin II inhibitor (blebbistatin), which directly targets actomyosin contractility (Fig.5 D, E). We found that these two inhibitors rescued the contractile phenotype induced by the loss of β4 and plectin, which suggests that a RhoA/ROCK/p-MLC signaling axis is involved in the regulation of cytoskeletal tension driven by the loss of β4 and plectin.

2. Comment: In the absence of β4 and in mutant cell lines, YAP is translocated to the nucleus. This is an interesting finding, however, no data are provided to investigate the corresponding mechanism and to link it to the overall phenotype. This should be experimentally addressed.

Response: As suggested by the reviewer, we further investigated how integrin β4 and plectin regulate the localization and activity of YAP by inhibiting the FAK- PI3K-AKT-mTOR and RhoA/ROCK/pMLC signaling pathways (Fig. 5 D, E). The obtained results (lines 284-301) provide evidence that integrin β4 suppresses FAK-PI3K and RhoA/ROCK/pMLC signaling, which subsequently results in the phosphorylation and inactivation of YAP.

3. Comment: Understanding how HD mediate the crosstalk with FA to control traction forces is key to the overall story. Plectin is implied to play a significant role in this but no molecular evidence is provided. This should be done.

Response: Plectin acts as a cross-linker that directly bridges keratin filaments not only to integrin α6β4, but also to the actin cytoskeleton. To evaluate its role in the crosstalk between HDs and FAs, we made use of keratinocytes expressing the integrin β4 mutant (β4-R1281W), which cannot bind plectin and thus cannot interact with the keratin intermediate filament network. In addition, we generated plectin-deficient keratinocytes. In both cell lines we observed an increase in actomyosin-mediated cellular tension (based on MLC phosphorylation). This increase in cellular tension was particularly prominent in the plectin-deficient cells (Fig. 4 F, G), which is most likely the result of the total disruption of the linkage between the actin and keratin network. Since we find similar effects on cellular tension in our integrin β4 mutant (β4-R1281W) expressing keratinocytes and in our plectin-deficient cells, we conclude that plectin is a key player in mediating the crosstalk between HDs and FAs to control cell tension and traction forces. Moreover, in our revised paper, we have investigated the activity of YAP and FAK-PI3K-AKT-mTOR pathway in both integrin β4 deficient and plectin-deficient PA-JEB/β4 cells and found similar results (Fig. 5 D, E, lines 284-301).

4. Comment: Following papers should be discussed. Correia et al., J Cell Biol. 1999 Aug 23;146(4):831-42 Seltmann et al., J Invest Dermatol. 2013 Jan;133(1):181-90. doi: 10.1038/jid.2012.256 Hiroyasu et al., FASEB J. 2016 Jun;30(6):2298-310. doi: 10.1096/fj.201500160R Pora et al., J Invest Dermatol. 2019 Apr 2. pii: S0022-202X(19)31446-0. doi: 10.1016/j.jid.2019.03.1139

Response: We have incorporated the mentioned publications in our revised manuscript in line 143-145 and 415-432.

Reviewer #3

1. Comment: There does not appear to be clear evidence of a physical linkage between HDs and FAs as indicated in the discussion and Fig 7. While this can be inferred by the known roles of plectin and the mechanical data presented here, it is an important unanswered question of exactly how and where the two are linked? Perhaps proximity ligation assays or IPs would be one way to address this question?

Response: We thank the reviewer for this useful remark. To address this issue we provide data from integrin β4 proximity biotinylation assays (BioID), showing that several FA components are found in close proximity of the integrin α6β4 (Fig.2A; Fig. S2).

We believe that these data, combined with the super resolution images in our study and work published by Pora et al. and Ozawa et al.(Pora et al., J Invest Dermatol. 2019 Apr 2. pii: S0022-202X(19)31446-0. doi: 10.1016/j.jid.2019.03.113; Ozawa et al., 2010. doi:10.1038/jid.2009.439.), confirm the physical linkage between FAs and HDs.

2. Comment: Why does the R1281W mutant not fully rescue cell spreading and force generation on the micropillar? Could this possibly indicate that plectin binding does not completely mediate force coupling between FAs and HDs? Perhaps analysis of PlecKO cells in this assay would be insightful?

Response: It is true that on soft pillars, PA-JEB cells expressing β4-R1281W do not fully rescue the phenotype. The β4-R1281W mutant behaves comparable to the β4-AD mutant on stiff pillars (the measured traction forces do not differ significantly from each other). As we discussed in the manuscript (lines 403-414), it is possible that there is a certain window for which the IF network is able to resist cellular contractility. IFs might only be stretched and oppose actomyosin contractility in response to high actomyosin-mediated cellular tension generated by cells seeded on stiff substratum. Another possible explanation is that the interaction of α3β1 and/or α6β1 with laminin-332 compensates for the loss of α6β4-mediated adhesion. Because these integrins can be incorporated into focal adhesions, more traction force might be generated in keratinocytes expressing the integrin β4-AD mutant. This is in line with the data presented in Fig. S1A and F, which show that impaired binding of both integrin α6β4 and α3β1 to laminin-332 leads to severe adhesion defects, while blocking adhesion of β4 alone has only a relatively minor effect on adhesion.

We have shown the p-MLC level in plectin KO cells is much higher than that in wild-type cells (Fig. 4 F, G), which is comparable to what we have seen for β4-R1281W cells. The dramatic effect of plectin deletion on cellular tension might be due to loss of keratin association with both actin filaments and integrin β4. In our revised manuscript, we have further investigated the pFAK and YAP activity, which are read-outs of actomyosin-mediated cellular tension, in plectin KO cells (Fig. 5 D,E).

3. Comment: The authors describe the role of HDs in modulating FA maturation, but only FA size is quantified. It would be good to include measurements of FA length and staining for additional FA components, such as paxillin or zyxin.

Response: We have performed the requested measurements of FA length and confirmed our findings by staining for paxillin (Fig. S1B-E).

4. Comment: Another key question is what are the effects of altered mechano-transduction via HDs on cell phenotype? Are there proliferative or migratory defects as might be affected by changes in FA maturation and YAP localisation.

Response: Lessons learned from patients show that mutations in the gene encoding integrin β4 (ITGB4) cause human blistering diseases, e.g. junctional epidermolysis bullosa with pyloric atresia (JEB-PA) and epidermolysis bullosa (EB) simplex with PA (McGrath, 2015; Fine et al., 2014). The β4-R1281W mutant is found in patients suffering from a non-lethal form of JEB, which indicates that although adhesion to the basement membrane still can take place, the connection of integrin β4 to the IF network is necessary for the proper functioning of the skin. This suggests that the integrity of the skin is greatly impaired and fragile upon the alteration of the cell's mechanical features. In our case, cells carrying integrin β4 mutants generate more mature FAs, higher contractility and spread more than cells expressing wild type β4.

We also presented *in vitro* wound healing assays in our revised manuscript. In these assays, we tracked focal adhesion and type I hemidesmosome components and found dissolved type I hemidesmosomes and an increase in cellular tension and assembly of mature focal adhesions (probed by phospho-paxillin) at the leading edge of migrating cells. These data, together with the findings described by Wozniak et al. (*Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1692:103–119. doi:10.1016/j.bbamcr.2004.04.007), indicate that FAs and higher tension are needed by migrating cells.

5. Comment: In the methods section it would be helpful to provide more detail on cell line generation and clonality rather than referring to previous publications? Similarly in the figure captions, a clearer description of the vector only or transfection controls would be helpful.

Response: We have modified the methods section and figure captions to provide a clearer explanation of the cell line generation and transfections (lines 446-514).

6. Comment: The line scan data is Fig 3 does not add much as it depends on how the lines are drawn and only represents cells in one image. The same qualitative

information can be observed from the IF images alone, and the quantitative measurement of fluorescence across many cells in 3C is more convincing.

Response: We have deleted the line scan data as indicated by the reviewer.

7. Comment: In Fig 4, it would be good to show a super-resolution image of the beta4(-) cells as well to see how association between IFs and FAs compares with beta4(+) cells.

Response: In our revised manuscript, we added the super-resolution images of β4-deficient cells for comparison in Fig.2C and D.

October 28, 2019

Re: JCB manuscript #201904137R-A

Prof. Arnoud Sonnenberg
The Netherlands Cancer Inst.
Div. of Cell Biology
Plesmanlaan 121
1066 CX Amsterdam 1066 CX
Netherlands

Dear Prof. Sonnenberg,

Thank you for submitting your revised manuscript entitled "Role of hemidesmosomes in cellular force regulation". The manuscript has been seen by the original reviewers whose full comments are appended below. The reviewers are overall positive about the work in terms of its suitability for JCB, however, following reviewer 1's comment we agree that you should improve your immunofluorescence data on migrating cells to better support your interpretation. The other comments are all minor and can be easily answered by some rewording.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Upon resubmission, please also follow these manuscript organization and formatting instructions:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the possible acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, * including inset magnifications. * Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* To better convey the advance of your study we suggest the following title:

"Hemidesmosomes modulate force generation via focal adhesions"

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Sincerely,

Sandrine Etienne-Manneville, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

The authors have made an effort to produce physiological evidence for their findings by immunostaining of migrating cells. I am not very happy with the quality of the images and the lack of quantification but acknowledge their attempt to put the data in a broader context. I think that the description of the images is somewhat biased as I do not see the compelling evidence, for example, for the conclusions that the expression and organization of type I HDs is very limited in cells at the wound edge or, conversely, for increased FA staining in cells at the wound edge. These statements should at least be toned down or must be further substantiated.

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

In this manuscript, the authors show that keratinocytes lacking hemidesmosomal integrin α6β4 exhibit increased focal adhesion formation, cell spreading and traction-force generation. This supports the view that HDs serve as regulators of cellular mechanical forces.
Overall, the effort by the authors has clarified the majority of issues and I feel that the manuscript is now much more solid than before. Among other data, there is BioID-based evidence for direct interactions between focal adhesion (FA) and hemidesmosome (HD) components. At the current

stage of analysis, one can't fully exclude that the interactions (about which I have no doubt as the controls seem to be done well) do not take place between mature FA and HD but between constituent proteins during exocytosis or recycling. The authors might want to consider this in their discussion.
In line 399-400, the authors discuss "Since IFs are elastic and can be stretched several times their original lengths without breaking...". This staining should be referenced. Also, IF stretching by such an extent was accompanied by thinning of filaments. If this has not been documented during the experiments, the statement should be worded cautiously.

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

The revised manuscript has addressed all comments raised and is now suitable for publication.

2nd Revision - Authors' Response to Reviewers: November 19, 2019

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

Q: The authors have made an effort to produce physiological evidence for their findings by immunostaining of migrating cells. I am not very happy with the quality of the images and the lack of quantification but acknowledge their attempt to put the data in a broader context. I think that the description of the images is somewhat biased as I do not see the compelling evidence, for example, for the conclusions that the expression and organization of type I HDs is very limited in cells at the wound edge or, conversely, for increased FA staining in cells at the wound edge. These statements should at least be toned down or must be further substantiated.

A: We thank the reviewer for the feedback on our revised manuscript. We understand the concern that the description of the wound healing experiments might seem somewhat biased. To address this, we present a new panel to Figure 1A (triple IF staining for pMLC, vinculin and F-actin) and added quantifications of the amount of type I HDs, phosphorylated MLC, and FAs (based on the vinculin staining) present in cells at the wound edge and in cells located further away from the wound (i.e. stationary versus leading cells).

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

Q: In this manuscript, the authors show that keratinocytes lacking hemidesmosomal integrin α6β4 exhibit increased focal adhesion formation, cell spreading and traction-force generation. This supports the view that HDs serve as regulators of cellular mechanical forces.
Overall, the effort by the authors has clarified the majority of issues and I feel that the manuscript is now much more solid than before. Among other data, there is BioID-based evidence for direct interactions between focal adhesion (FA) and hemidesmosome (HD) components. At the current stage of analysis, one can't fully exclude that the interactions (about which I have no doubt as the controls seem to be done well) do not take place between mature FA and HD but between constituent proteins during exocytosis or recycling. The authors might want to consider this in their discussion.
In line 399-400, the authors discuss "Since IFs are elastic and can be stretched several times their original lengths without breaking...". This staining should be referenced. Also, IF stretching by such an extent was accompanied by thinning of filaments. If this has not been documented during the experiments, the statement should be worded cautiously.

A: We are pleased to read that the additional experiments could convince the reviewer of the interaction between FA and HD components. We agree with the reviewer that we cannot conclude from the BioID-based evidence that there is a direct physical link between these components. That is also why when we make this claim in our manuscript, we always base this conclusion on the BioID data and the super resolution microscopy images.

Regarding the stretching of the IFs, we understand the possible confusion caused by this sentence. We should have provided the corresponding reference (Charrier and Janmey, 2016) in this sentence (which we did in the latest version of the manuscript). Additionally, we made some minor changes to the text to make clear what conclusions were drawn from our data and what is hypothesized, and/or presented in our summarizing model in Fig. 8.