



Induction of ligand promiscuity of α V β 3 integrin by mechanical force

Michael Bachmann, Markus Schaefer, Vasyl V. Mykuliak, Marta Ripamonti, Lia Heiser, Kai Weissenbruch, Sarah Krübel, Clemens M. Franz, Vesa P. Hytönen, Bernhard Wehrle-Haller and Martin Bastmeyer

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Original submission

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MS TITLE: Induction of ligand promiscuity of α V β 3 integrin by mechanical force

AUTHORS: Michael Bachmann, Markus Schaefer, Vasyl V. Mykuliak, Marta Ripamonti, Lia Heiser, Kai Weissenbruch, Sarah Kruebel, Clemens M. Franz, Vesa P. Hytönen, Bernhard Wehrle-Haller, and Martin Bastmeyer

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 raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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

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

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors meticulously demonstrated that α V β 3 integrin preferentially bind vitronectin over fibronectin under all conditions. Importantly, the binding to vitronectin is less

force-dependent than the binding to fibronectin, which allows the $\alpha V\beta 3$ integrin to adjust ligand specificity according to physical environment. The authors also generated new tools to tune the conformation of $\alpha V\beta 3$ integrin which could be useful for the community.

The overall quality of the data is high and the scientific conclusion is sound. Most of the concerns from the previous review have been sufficiently addressed in my opinion. A lot of technical concerns raised up by reviewer 2 and 3 from the previous round cannot be fully explained without detailed structural interrogation, which is beyond the scope of this manuscript.

Comments for the author

Minor issues:

1. The authors assume central adhesions are under low force when interpreting data from Fig.5. The assumption that central $\alpha V\beta 3$ integrin-containing adhesions are under low force like central $\beta 1$ integrin containing-adhesions is not valid unless measured with a tension sensor. I would suggest to move the rationalization in the result part to discussion.

2. In Fig.5c the peripheral FA is not obvious for WT $\alpha V\beta 3$ integrin yet it was measured to be prevalent in Fig.5d. The authors should co-stain with Pxn and image the cells under TIRF for Fig.5c to minimize the interference from signals in ER.

3. To further corroborate the notion that force-induced swing-out facilitate $\alpha V\beta 3$ -FN binding, the authors should compare N305T mutant with wild type integrin in the hydrogel experiment if time permits.

Minor error:

Fig.5d, colours in graph and legend do not match.

Reviewer 2

Advance summary and potential significance to field

This paper shows that $\alpha V\beta 3$ binds preferentially to Vn at low cell contractility and requires high cell contractility to swing out the b hybrid domain enabling Fn binding

Comments for the author

The study by Bachmann et al addresses the question what determines binding of $\alpha V\beta 3$ integrin, which binds to several ligands, to a certain ligand. Using binary-choice substrates they show that $\alpha V\beta 3$ integrins prefer binding to vitronectin over fibronectin under various in vitro conditions. However, force-mediated hybrid domain swing out facilitates fibronectin binding by $\alpha V\beta 3$. This is an elegant, comprehensive study. The data are of high quality, well described and discussed. The manuscript is well suited for publication at JCS.

I only have minor points:

#The different manipulations/conditions only result in rather weak changes for ligand selectivity, which might be due to the residual expression of endogenous $\beta 3$ integrin. This point should be discussed.

#please provide an integrin profile of the used cell lines

#Fig 3a-d the activating mutations caused the formations of central $\alpha V\beta 3$ clusters. These clusters contained only talin but no vinculin, paxillin or actin fibers. Is kindlin-2 present, as it was suggested to be important for integrin clustering?

#the authors claim that the GD25 cells actively migrate towards Vn. To my opinion this is a misinterpretation as the cells do not follow a chemotactic cue rather than accumulate over time at the site/ligand which they prefer to bind to.

First revision

Author response to reviewers' comments

Response Letter by Bachmann et al.

Please find our responses in italic letters below and text changes in the manuscript due to reviewer comments printed in red here as well as in the manuscript itself.

We would like to thank the editor and all reviewers for their positive, encouraging comments and for acknowledging the quality of our work.

Reviewer 1 Advance Summary and Potential Significance to Field:

In this manuscript, the authors meticulously demonstrated that α V β 3 integrin preferentially bind vitronectin over fibronectin under all conditions. Importantly, the binding to vitronectin is less force-dependent than the binding to fibronectin, which allows the α V β 3 integrin to adjust ligand specificity according to physical environment. The authors also generated new tools to tune the conformation of α V β 3 integrin which could be useful for the community.

The overall quality of the data is high and the scientific conclusion is sound. Most of the concerns from the previous review have been sufficiently addressed in my opinion. A lot of technical concerns raised up by reviewer 2 and 3 from the previous round cannot be fully explained without detailed structural interrogation, which is beyond the scope of this manuscript.

Reviewer 1 Comments for the Author: Minor issues:

1. The authors assume central adhesions are under low force when interpreting data from Fig.5. The assumption that central α V β 3 integrin-containing adhesions are under low force like central β 1 integrin containing-adhesions is not valid unless measured with a tension sensor. I would suggest to move the rationalization in the result part to discussion.

While preparing this manuscript we published a study about a new GFP-labeling strategy for β 1 integrins and differences concerning the splice isoforms β 1A and β 1D (Soto-Ribeiro et al., 2019). This study also indicated to us that dynamics (and potentially tension status) of integrin adhesions cannot be easily judged exclusively based on subcellular localization. This might be especially true for central adhesions with a potential mixture of focal and fibrillar adhesions (or even reticular adhesions). Therefore, we agree with this reviewer that tension sensors (traction force microscopy, FRET-based talin or RGD tension sensors, AFM) would be needed to fully confirm our interpretation of this experiment. However, establishing this experiment would be too time consuming within the time frame of this revision. Given that this experiment therefore offers no immediate, clear insights we decided in accordance with this reviewer's suggestions to remove this experiment from the manuscript. Importantly, we believe that removing these data does not diminish the findings of this manuscript. We hope that this decision finds the approval of this reviewer.

2. In Fig.5c the peripheral FA is not obvious for WT α V β 3 integrin, yet it was measured to be prevalent in Fig.5d. The authors should co-stain with Pxn and image the cells under TIRF for Fig.5c to minimize the interference from signals in ER.

We agree that TIRF microscopy would be better suited to quantify adhesions for this experiment. However, as explained above, we think that the insights we could gather from this experiment would still be limited and accordingly decided to remove this experiment.

Based on this reviewer's suggestion #3 we added new data (see below and Fig. R1) that (we believe) is better suited for this manuscript. We used again epifluorescence and not TIRF in that experiment. As explained below, this was necessary due to the high number of conditions. To avoid problems with strong background fluorescence in the cell center, we restricted the analysis for Fig. R1 to peripheral adhesions. This approach should enable a reliable quantification and avoid the problem correctly raised by this reviewer for former Fig. 5C, D.

3. To further corroborate the notion that force-induced swing-out facilitate α V β 3-FN binding, the authors should compare N305T mutant with wild type integrin in the hydrogel experiment if time permits.

We thank this reviewer for this suggestion which would indeed help to support the relevance of hybrid domain swing-out for α VB3 integrin - Fn binding. We performed initial tests but unfortunately failed to perform this experiment in a reliable, quantifiable way for the following reasons:

- The author responsible for the hydrogel experiments moved in the meantime to Geneva. Unfortunately, confocal microscopes at the bioimaging facility in Geneva are currently not equipped with a long- distance high-resolution objective that was used for the initial experiments to image focal adhesions on hydrogels. Therefore, we failed to image focal adhesions in a way that enables us to quantify adhesion maturation as done for the former hydrogel experiment (former Fig. 5E-F).
- The initial experiments used GD25 cells that do not express β 1 integrin and rely on endogenous α VB3 integrin for cell adhesion. Overexpressed α VB3-N305T would have to compete with endogenous α VB3 integrin. Measuring cell size (as in former Fig. 5G) was therefore not feasible as readout for α VB3-N305T while measuring focal adhesion size was prevented by the lack of a suitable objective as mentioned above.

Given these obstacles we tried to set up an alternative experiment addressing this reviewer's concern. We decided to transfect NIH3T3 cells (virtually no endogenous α VB3; see Fig. R2) expressing either α VB3-wt or α VB3-N305T on Fn or Vn coated cover slips and in presence of different amounts of a contractility inhibitor (ROCK-inhibitor Y27632). Cells were cultured in the absence of serum to avoid contamination of the substrate with serum-Vn. Since we used a clone of NIH3T3 cells that expresses almost no α VB3 integrin at the cell surface (see also Fig. R2 and Fig S1A; used throughout this study) we can propose that α VB3-N305T does not compete with endogenous α VB3 integrin in these cells. However, endogenous α 5B1 integrin is present, meaning that our analysis had to be restricted to β 3-wt GFP or β 3-N305T GFP signals and that cell size measurements would be not informative due to cell spreading via endogenous α 5B1.

We used four different concentrations of Y27632 (0 μ M, 1 μ M, 5 μ M, 10 μ M; please note that 10 μ M Y27632 was also used on Fn/Vn substrates as shown in Fig. 2B, G) and analyzed the average focal adhesion size per cell, which we used as a marker for adhesion maturation. Conditions were blinded during analysis to ensure unbiased analysis of adhesion size (Fig. R1; see figure legend for details).

As you can see, α VB3-wt has only a limited ability to establish focal adhesions on Fn (Fig. R1C). This probably explains the lack of effect of different Y27632 concentrations (Fig. R1A). α VB3-N305T on the other hand, is able to establish focal adhesions on Fn under control conditions but shows a Y27632-concentration dependent reduction in focal adhesion size until reaching the levels obtained with α VB3-wt levels at 5-10 μ M Y27632.

In cells cultured on Vn (Fig. R1B), α VB3-wt establishes focal adhesions similar to α VB3-N305T. However, a constitutive hybrid domain swing-out (N305T) seems to limit the effect of contractility inhibition for binding to Vn. The data show considerable variation. Given the number of conditions, we had to restrain from TIRF microscopy and used mounted samples and epifluorescence microscopy which might contribute to this variation of data points. However, we believe that the data is robust enough to confirm again that

- α VB3-integrin is primarily a Vn-binding integrin (compare α VB3-wt in R1A vs. R1B),
- that hybrid domain swing-out is essential for Fn-binding while somewhat less relevant for Vn-binding (compare α VB3-N305T in R1A vs R1B),
- and that α VB3-N305T in absence of force is not a maximally activated integrin (compare R1A and manuscript Fig. 3E, G).

Importantly, this experiment extends our observations from Fn/Vn substrates to homogenous substrates and confirms our model for force-mediated Fn-binding of α VB3-integrin in an independent experimental setting.

Given a word limit for JCS articles we decided to show only Fig. R1A in the manuscript. We also assume that this part of the experiment serves best to address the point raised by this reviewer.

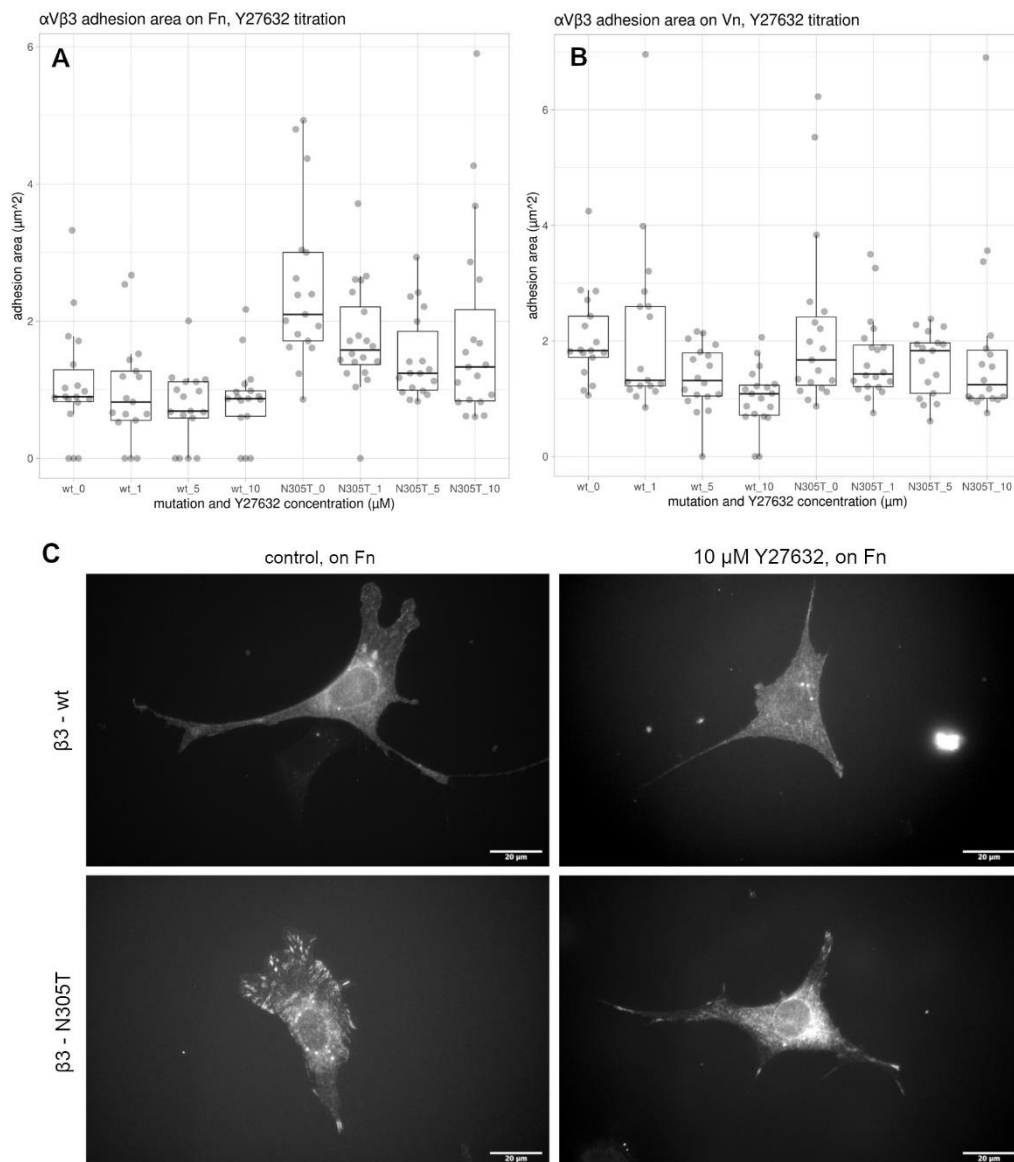


Figure R1: NIH3T3 cells were transfected with B3-wt GFP or B3-N305T GFP and cultured in DMEM without serum for 4 hrs in presence of the ROCK inhibitor Y27632 with increasing concentration as indicated. Cover slips were coated with Fn (10 μg/ml; Fig. R1A and R1C) or Vn (5 μg/ml; Fig. R1B). Cells were fixed and images were taken with 63X/1.4NA oil immersion objective. Focal adhesion area was analyzed with Fiji in the following way: background subtraction with rolling ellipsoid; analysis of peripheral adhesions to avoid background signal in cell center; pictures were thresholded and “analyze particles” with area threshold “0.5 μm² - infinity” was used to restrict analysis to focal adhesions and to avoid including noise into the analysis; therefore, 0 μm² indicates that no B3-adhesion reached a size of 0.5 μm² or bigger; one data point represents the average area size of focal adhesions within one cell. $N = 3$ and number of analyzed cells $n \geq 17$.

Minor error:

Fig.5d, colours in graph and legend do not match.

We excuse for this mistake. As mentioned above, we deleted this graph from the manuscript which should solve this issue.

Reviewer 2 Advance Summary and Potential Significance to Field:

This paper shows that α V β 3 binds preferentially to Vn at low cell contractility and requires high cell contractility to swing out the b hybrid domain enabling Fn binding

Reviewer 2 Comments for the Author:

The study by Bachmann et al addresses the question what determines binding of α V β 3 integrin, which binds to several ligands, to a certain ligand. Using binary-choice substrates they show that α V β 3 integrins prefer binding to vitronectin over fibronectin under various in vitro conditions. However, force-mediated hybrid domain swing out facilitates fibronectin binding by α V β 3. This is an elegant, comprehensive study. The data are of high quality, well described and discussed. The manuscript is well suited for publication at JCS.

I only have minor points:

#The different manipulations/conditions only result in rather weak changes for ligand selectivity, which might be due to the residual expression of endogenous β 3 integrin. This point should be discussed.

#please provide an integrin profile of the used cell lines

We thank the reviewer for raising this relevant point. We added additional flow cytometric data of the cell lines used in this study and extended our previous figure concerning this point (Fig. R2; R2A is also shown in the manuscript as Fig. S1A). As the data indicates (Fig. R2A, B), the NIH3T3 subclone used in this study barely expresses any surface α V β 3 integrin. Thus, expressed β 3 mutants do not compete with endogenous α V β 3 integrin (Fig. R1A; expressed β 3 integrins showed two order of magnitudes higher surface staining).

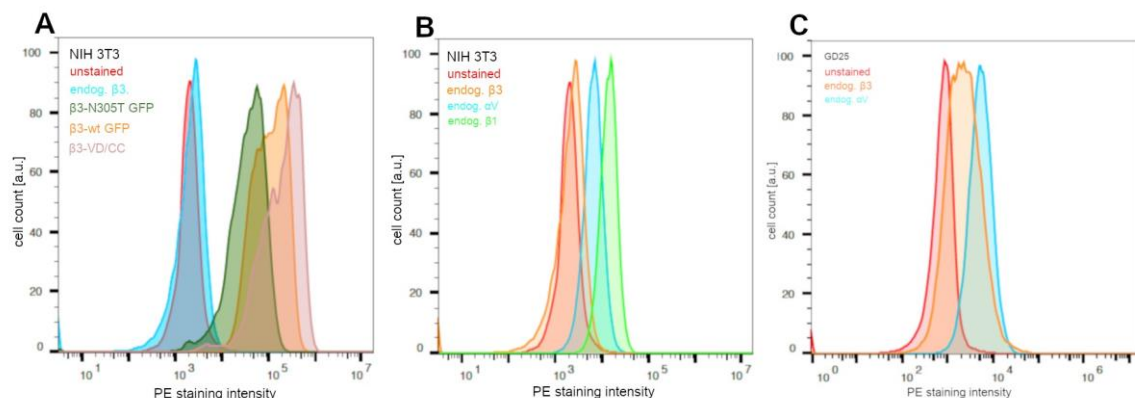


Fig. R2: NIH3T3 cells (Fig. R2A, B) or GD25 cells (Fig. R2C) were stained with primary antibodies against β 3 integrin (clone HMB3-1, BD #550541, 1:500), α V integrin (invitrogen, clone RMV-7, #14-0512-85, 1:500), or β 1 integrin (clone Hmb1, 1:500) followed by a staining with PE labeled secondary antibodies (anti-hamster PE: Jackson ImmunoResearch, #127-115-160, 1:600; anti-rat PE: Jackson ImmunoResearch, #112-116-143, 1:800). PE intensity was analyzed with an Accuri 6 flow cytometer. NIH3T3 cells were mock transfected for conditions described as unstained or for measuring endogenous integrin levels. For these conditions, cells were gated for viability with SSC/FSC signals. NIH3T3 cells transfected for α V β 3 integrin variants (Fig. R2A) were additionally gated for positive GFP-signal. At least 10 000 cells were analyzed.

Concerning the rather weak changes in ligand selectivity, we want to point to the 0-100% scale we used to quantify ligand selectivity and what we could expect from α V β 3 integrin as a possible change in ligand selectivity:

- A lower limit is set by noise. Even when pictures indicated an exclusive preference for Vn (Fig. 2A, B, E, Fig. 3E, Fig. 4 F, G, Fig. S3 F-I) we quantified a colocalization with Fn around roughly 5%.
- A Fn colocalization above 50% (meaning that α V β 3 starts to prefer Fn over Vn) would require conformational changes that invert the initial preference of α V β 3 integrin. We believe that this is a very unlikely scenario and would require surprising rearrangements in the binding

pocket.

Given these restrictions, we could have adjusted the y-scale in our quantifications by applying offsets and a normalization leading to apparently stronger changes in ligand selectivity. However, we felt that this approach would be misleading in understanding the dynamic behavior of α V β 3 integrin in the presence of two ligands at the same time. As an alternative, we now mentioned fold-changes in Fn colocalization in the manuscript in order to highlight the extent of changes in ligand selectivity of α V β 3 integrin and to address the issue raised by this reviewer.

#Fig 3a-d the activating mutations caused the formations of central α V β 3 clusters. These clusters contained only talin but no vinculin, paxillin or actin fibers. Is kindlin-2 present, as it was suggested to be important for integrin clustering?

Some of us (Wehrle-Haller lab) submitted an extensive paper dealing with activation of integrins by talin1 and kindlin2 that addresses this question in detail. In fact, kindlin2 is present in these α V β 3 integrin clusters. However, to answer this reviewer's question directly we performed this experiment with NIH3T3 cells expressing β 3-wt GFP and kindlin2 mCherry (Fig. R3). This experiment confirmed that kindlin2 is present in Mn²⁺ induced clusters in the experimental system used in this manuscript.

“NOTE: We have removed unpublished data that had been provided for the referees in confidence”.

#the authors claim that the GD25 cells actively migrate towards Vn. To my opinion this is a misinterpretation as the cells do not follow a chemotactic cue rather than accumulate over time at the site/ligand which they prefer to bind to.

We performed the experiments for Fig. 5A, B in absence of serum to avoid contamination of surface coating by serum-Vn. This also drastically slowed down any cell migration hampering the interpretation of this experiment in terms of cell migration. Therefore, we were also hesitant to speak about a typical migration from Fn to Vn and tried to emphasize this by a respective wording when addressing experiment Fig. 5A, B.

We realize that this intention was not obvious enough and changed our description of the experiment accordingly (changes in red; line 402-405):

“To understand how cell **behavior** is influenced when GD25 cells can choose between Vn and Fn, we produced striped patterns of Vn/Fn with cellular resolution (Vn: 20 μ m stripe width; Fn: 40 μ m). Live cell imaging for 12 hrs on these Fn/Vn stripe patterns revealed a **turning** of cells **away** from Fn towards Vn (Fig. 5A and Video S8).”

Line 409-410:

“With increasing time, the surface area of single cells colocalized less with Fn (cell/Fn colocalization after 8 hrs: 28.4%; 24 hrs: 14.6%) demonstrating a preference to **adhere to Vn**.”

At the same time, however, migration of GD25 cells on homogenous Fn or Vn in presence of 1% FCS (shown in Supplementary Movie 7) showed different migration velocities on the respective ligands ($v_{Fn} = 12.0 \pm 3.08$ μ m/h, $v_{Vn} = 6.7 \pm 0.39$ μ m/h). Therefore, we considered it justified to say (line 94f):

“We further show that these ligand-binding properties modulate cellular behavior during spreading, **migration**, and mechanotransduction depending on the respective ECM protein.”

We hope the reviewer agrees with our reasoning and our approach to address the issue raised by her/him.

References:

Soto-Ribeiro, M., Kastberger, B., Bachmann, M., Azizi, L., Fouad, K., Jacquier, M. C., . . . Wehrle-Haller, B. (2019). β 1D integrin splice variant stabilizes integrin dynamics and reduces

integrin signaling by limiting paxillin recruitment. *J Cell Sci*, 132(8). doi:10.1242/jcs.224493

Second decision letter

MS ID#: JOCES/2019/242404

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ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

This manuscript provides convincing evidence that ligand preference of $\alpha V\beta 3$ integrin is mechanically regulated.

Comments for the author

The authors have largely resolved my concerns in the revised manuscript. The work is technically sound and should be published and evaluated by the integrin community.

Reviewer 2

Advance summary and potential significance to field

I am satisfied with the reviewers comments and I congratulate them to this excellent study.

Comments for the author

I am satisfied with the reviewers comments and I congratulate them to this excellent study.