Precise coordination of cell-ECM adhesion is essential for efficient melanoblast migration during development

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MS TITLE: Precise Coordination of Cell-ECM Adhesion is Essential for Efficient Melanoblast Migration During Development

AUTHORS: Amanda Haage, Kelsey Wagner, Caitlin Mitchell, Katharine Goodwin, Aaron Bogutz, Louis Lefebvre, Catherine D Van Raamsdonk, and Guy Tanentzapf

I apologize for the very long delay before coming back to you. However, I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is clearly very positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Haage et al investigate the role of cell-ECM adhesion during the long-range migration of melanoblasts. Integrin-mediated adhesion has been difficult to study in melanoblast migration because of functional redundancy, so the authors employ a previously generated autoinhibition-defective mutant in Talin1 (Tln1 E1770A) to explore this question. They show that mice homozygous for the Tln1 E1770A mutation display pigmentation defects along the ventral axis and in the extremities of the animals suggesting defective melanoblast migration. By monitoring the distribution of melanoblasts across the embryo, they show that the dorsal to ventral population of the skin with melanoblasts is delayed in Tln1 E1770A mutants. This was not due to reduced

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melanoblast division, and cell shape analyses pointed to a possible defect in cell migration. To determine a cell autonomous role for Talin in melanocyte migration, the authors cultured primary melanocytes from Tln1 E1770A animals, which displayed similar defects in morphology. This was not due to differences in actin protrusions, alignment or bundling but correlated with increased focal adhesion size strength, and active integrin levels. In skin explants derived from Tln1 E1770A mutants melanoblasts showed clear defects in migration speed, persistence, and displacement. They further used a melanoblast-specific conditional Itga6 mutant to suggest a function for integrin receptors in long-range melanoblast migration.

Overall, the manuscript was clearly written and the data support a role for cell-ECM adhesion in melanoblast migration. The strongest piece of data is the cell migration analysis in skin explants. In the striking supplemental movies provided, melanoblasts from Tln1 E1770A mutants display truncated protrusions and very little cell body displacement compared to controls. Unfortunately, the cell shape and protrusion analyses of fixed tissue sections, and low resolution images of LacZ stained embryos, were much less convincing.
Moreover, because the Tln1 E1770A mutation affects all cells of the embryo, it is difficult to conclude the migratory defect in Tln1 E1770A mutants is due to changes in integrin-mediated adhesion in the melanocytes themselves as opposed to nonautonomous changes in the substrate or environment.

Comments for the author

Specific comments
- Figure 3E-L. The low magnification, LacZ images and DCT-stained tissue sections used for quantifying cell shape do not show the striking defects observed in the live explant data. Because many protrusions will be out of the plane of sectioning, and because LacZ imaging doesn't provide the resolution of fluorescent labels images at high mag, re-analyzing cell shape and protrusion lengths using live explant images would really strengthen this data.

- Figure 7H-K. Similarly, the cell shape analysis performed on integrin cKO embryos should be improved. Considering how beautiful the live imaging data is using a Cre and tdTomato marker (both migration and cell shape), carrying out the cell shape analysis on fixed whole mounts expressing Mitf-Cre driving tdTomato would more accurately capture the cell shapes and be more convincing. If the genetics are too complicated, choosing another melanoblast antibody that can be imaged by immunofluorescence rather than LacZ should help reduce some of the variation and strengthen the characterization of what appears to be a very subtle phenotype.

- Further quantification of cell dynamics from the live movies in the Talin mutant could reveal more specific defects in migration that could bolster the findings. How do the cell shapes change over time? How long do protrusions persist? How dynamic are the protrusions. This analysis can be performed on the movies the authors already have.

Minor points:
- Figure 3A, C-the overlap between Edu and DCT is difficult to see. Please include all data points for box and whisker plots.
- Figure 4- Please include all data points for box and whisker plots. The actin in the 2D Fourier Transform is not visible. Is blank space being analyzed?
- Figure 5. Please include Y axis for E.
- Scale bars are missing throughout the figures
- Please indicate what steps are taken to ensure melanocyte cultures are not contaminated with other cell types. Is a cell type-specific marker used to co-stain for each experiment? Since the authors have already shown that Tln1 E1770A fibroblasts display enlarged focal adhesions, it is important to validate melanocyte cultures.

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Reviewer 2

Advance summary and potential significance to field

This study interrogates the role of integrin-mediated adhesion, as regulated by talin, in melanoblast migration from the neural crest through the dermis and epidermis to populate the mouse skin and hair follicles. The authors express an autoinhibition defective talin mutant in melanoblasts and study their migration and population of the embryo skin/hair during development. They also delete ITGA6 and show that although this does not lead to pigmentation defects, there are shape changes in melanoblasts in embryos, suggesting a subtle role in migration. Their study highlights the use of tools, such as mutant talin, to unpick the role of adhesion during melanoblast migration. The data are beautifully presented and they make a compelling case for the importance of integrin-based adhesion in melanoblast migration during development. There are a few places where important controls should be considered – e.g. showing that the mutant talin is expressed and also whether ITGA6 is expressed in their target cells. Additonally, the important question of whether these perturbations affected association with or crossing of the basement membrane was not addressed.

Comments for the author

Specific Points:
1. It would be nice to see a western blot showing talin and ITGA6 expression in the cultured melanoblasts- both from the control and mutant mice. This would give an idea of at least relative levels of expression of each protein and whether the mutant talin was overexpressed or underexpressed in the melanoblasts. It would also validate that the mutant protein was expressed- even if it is not possible to see whether it is mutated – as opposed to say just being degraded by the melanoblasts/cytes.
2. Figure 5H- I was confused by the n numbers from the y-axis on the graph. It looks like 13,57 and 18,751 ?
3. In Figure 3, the authors use skin sections to count cells positive for DCT and EDU. If they were able to stain similar sections for ColIV, they could quantify the proportion of cells attached to / above/ below the basement membrane. While I don't think that this is absolutely required, it is a great opportunity to see if the talin mutant affects bm adhesion in vivo and in my opinion it would greatly strengthen the argument that the authors make around this.

Reviewer 3

Advance summary and potential significance to field

This study from the Tanentzapf lab is investigating how the intracellular integrin binding protein Talin regulates the migration of 'melanoblasts' during skin development in the mouse model. Elegant in vivo experiments using multiple genetic models demonstrate that melanoblasts autonomously require Talin's auto-inhibition function to normally populate the skin and the hair follicles during development. Mechanistically, the authors show that an auto-inhibition block in Talin results in too strong adhesion of the melanoblasts and thus in defective migration leading to aberrant melanoblast distribution in adult mice.

This manuscript is a follow up study of earlier work by the same lab (Haage et al. 2018), however, it investigates a different cell population and leads to interesting new insight into the role of Talin during a complex migration pathway, the one of melanoblasts in the mouse skin. It provides convincing in vivo evidence that the auto-inhibition function of Talin is required to control cell-matrix adhesion during the migration process. Hence, it will be of significant interest to the integrin and adhesion community.

Comments for the author

Minor points.
1. I could not find any information in the methods section how the actin cytoskeleton in Figure 4G-I has been quantified. Please provide details.

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2. Figure 5E is missing the legend of the X-axis. As a different number of focal adhesions have been quantified in wild type versus TalinE1770A plotting the percentage of FAs in the different bins might be more appropriate.

3. I suggest to fuse Figure 6G and H as they are directly comparable.

4. Typo on page 19: ‘To directly address this point directly, we used…’

5. Structural support for the mechanism of Talin auto-inhibition has been very recently published and could be discussed (Dedden et al. Cell 2019).

First revision

Author response to reviewers' comments

Specific points:

1. “Figure 3E-L. The low magnification, LacZ images and DCT-stained tissue sections used for quantifying cell shape do not show the striking defects observed in the live explant data. Because many protrusions will be out of the plane of sectioning, and because LacZ imaging doesn’t provide the resolution of fluorescent labels images at high mag, re-analyzing cell shape and protrusion lengths using live explant images would really strengthen this data.”

We agree with the reviewer that this point should have been strengthened by additional analysis, and we followed up on their suggestion. In line with the reviewer’s comments we have now added additional analysis of cell shape that was carried out in of our explant movies (shown in in Figure 6 C-E). Thus, the conclusion that disrupting talin autoinhibition results in a less motile phenotype is backed from evidence in E15.5 trunk embryo sections (Figure 3), intact E15.5 fixed trunk skin (Supplemental Figure 1), and intact E15.5 live trunk skin (Figure 6).

2. “Figure 7H-K. Similarly, the cell shape analysis performed on integrin cKO embryos should be improved. Considering how beautiful the live imaging data is using a Cre and tdTomato marker (both migration and cell shape), carrying out the cell shape analysis on fixed whole mounts expressing Mitf-Cre driving tdTomato would more accurately capture the cell shapes and be more convincing. If the genetics are too complicated, choosing another melanoblast antibody that can be imaged by immunofluorescence rather than LacZ should help reduce some of the variation and strengthen the characterization of what appears to be a very subtle phenotype.”

We agree that the experiment suggested by the reviewer would be the ideal one and we very much wanted to add this to the manuscript. The genetics for doing the requested experiments with the Itga6∆MEL/∆MEL mice were not straightforward but we may have been able to do in time for the deadline if not for the Covid pandemic interfered and we had to stop all experiments and euthanize all but our essential stocks. Unfortunately, this means that doing this experiment will not be possible in the foreseeable future. We also tried a few antibodies but were not successful. Nonetheless, in line with the reviewer’s valid criticism we have weakened some of the language in the summary claim at the end of the “Integrins contribute to melanoblast migration” results section.

3. “Further quantification of cell dynamics from the live movies in the Talin mutant could reveal more specific defects in migration that could bolster the findings. How do the cell shapes change over time? How long do protrusions persist? How dynamic are the protrusions. This analysis can be performed on the movies the authors already have.”

We thank the reviewer for these suggestions and have now added much additional analysis of cell dynamics in our skin explant movies, hopefully maximizing the utility of our timelapse imaging

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experiments. We were able to confirm the shape analysis, providing much support for the claims made in Figure 3 and 4 as discussed above. We were also able to analyse and quantitate melanoblast shape changes and protrusive activity over time. The allowed us to demonstrate an overall decrease in cell dynamics in our mutants, as well as more mechanistic detail in how these shape changes translate to decreased motility. This has added a full additional figure (Figure 7), as well as panels C-E in Figure 6. These results are discussed mostly in an additional second paragraph to the “Talin autoinhibition regulates migration speed and persistence in melanoblasts” results section.

4. “It would be nice to see a western blot showing talin and ITGA6 expression in the cultured melanoblasts- both from the control and mutant mice. This would give an idea of at least relative levels of expression of each protein and whether the mutant talin was overexpressed or underexpressed in the melanoblasts. It would also validate that the mutant protein was expressed- even if it is not possible to see whether it is mutated – as opposed to say just being degraded by the melanoblasts/cytes.”

In line with the reviewer’s suggestion we have added a western blot analysis of talin expression in wildtype and in mutant E1770A TLN-1 neural plates. (Figure 1L). The level of mutant talin expression appears comparable to wildype talin expression. We had also previously done this in a more quantitative way with qRT-PCR in primary mouse embryonic fibroblasts derived from this same mutant line (Haage et al. 2018). We were unable to complete this analysis specifically with primary melanocytes due to low protein yield and limited cultures. Unfortunately, we were never able to establish proliferative primary melanocytes from either wild-type or mutant neonatal skin. Therefore, we used neural plates from E8.5 embryos since melanoblasts are of neural crest origin.

5. “Figure 5H- I was confused by the n numbers from the y-axis on the graph. It looks like 13,57 and 18,751 ?”

The n numbers for Figure 5 H represent individual FAs from across > 80 cells per genotype. The ratio of pixel intensity for the active integrin channel over total integrin channel is calculated per FA above an area threshold. The previous figure legend was incorrect in describing what the n value represented in this panel, this has now been fixed.

6. “Figure 3, the authors use skin sections to count cells positive for DCT and EDU. If they were able to stain similar sections for ColIV, they could quantify the proportion of cells attached to / above/ below the basement membrane. While I don’t think that this is absolutely required, it is a great opportunity to see if the talin mutant affects bm adhesion in vivo and in my opinion it would greatly strengthen the argument that the authors make around this.”

We thank the reviewer for this suggestion. We were able to generate trunk sections of both wildtype and mutant E15.5 embryos and stain them for ColIV and Dct. These are now included as Figures 3 J-L. We have used this data to replace the previous shape analysis done on intact embryo skin with LacZ stained melanoblasts, which has now been moved to Supplemental Figure 1. This new data demonstrates a normal basement membrane organization and adhesion by melanoblasts in our mutants. This helps further point the cause of pigmentation defects to be melanoblast specific cell migration defects from lack of talin autoinhibition.

Minor points:
1. “I could not find any information in the methods section how the actin cytoskeleton in Figure 4G-I has been quantified. Please provide details.”

We have now updated the “Primary Melanocyte Imaging and Automated Analysis” section of the materials and methods to include more detail from our previous manuscript (Haage et al. 2018). It now reads as follows “Fixed cell imaging and analysis was completed as previously described (Haage et al., 2018). Briefly, FAs, cell morphology, and actin fibers were analyzed using custom MATLAB protocols. To quantify actin fibers specifically we adapted previously used methods from Cetera et al. 2014 to calculate a 2D Fourier Transform across each cell in overlapping windows. Fibrousness is then based on the aspect ratio of this Fourier transform.”

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2. “Figure 5E is missing the legend of the X-axis. As a different number of focal adhesions have been quantified in wild type versus TalinE1770A plotting the percentage of FAs in the different bins might be more appropriate.”

Each panel in Figure 5E – F now have their own X and Y axis labels. We have replotted this data according to the reviewers’ suggestions. Figure 5E is now the percent of FAs by area. We also updated the numbers above each bar in Figure 5F to FA percent instead of individual FA number. These changes are reflected in the text and figure legends.

3. “I suggest to fuse Figure 6G and H as they are directly comparable.”

We have now plotted the average mean square displacement for both wildtype and mutant cells on the same axes, now Figure 6H

4. “Typo on page 19: ‘To directly address this point directly, we used…’”

We have now corrected this typo.

5. “Structural support for the mechanism of Talin auto-inhibition has been very recently published and could be discussed (Dedden et al. Cell 2019).”

We thank the reviewer for pointing out this important reference that we have now added to the second paragraph of our introduction.

6. “Figure 3A, C-the overlap between Edu and DCT is difficult to see. Please include all data points for box and whisker plots.”

In order to increase visibility of the overlap of stains represented in Figure 3A-D, we have decreased the DAPI blue channel. We have also added yellow and white triangles to indicate melanoblasts with and without Edu incorporated respectively. We also replotted Figures 3G-I to contain all individual data points.

7. “Figure 4- Please include all data points for box and whisker plots. The actin in the 2D Fourier Transform is not visible. Is blank space being analyzed?”

We have replotted Figures 4B-E, and I-J to contain all individual data points. To increase the visibility of phalloidin staining we increased the brightness of images in Figures 4A, and G-H. The 2D Fourier transform windows are generated by the mask of the cell. A window without prominent actin staining will yield a low aspect ratio Fourier transform, indicating low fibrousness.

8. “Figure 5. Please include Y axis for E”

Each panel in Figure 5E – F now have their now X and Y axis labels.

9. “Scale bars are missing throughout the figures”

We thank the reviewer for pointing out this oversight. Scale bars have now been added to Figure 1 A-F, K-N, Figure 2 A-B, D, F, H, J, Figure 3 A-D, F, J-K, Supplemental Figure 1A, Figure 8 A, C, E, H.

10. “Please indicate what steps are taken to ensure melanocyte cultures are not contaminated with other cell types. Is a cell type-specific marker used to co-stain for each experiment? Since the authors have already shown that Tln1 E1770A fibroblasts display enlarged focal adhesions, it is important to validate melanocyte cultures.”

We would like to draw the reviewer’s attention to the following section that was included in the previous version of the manuscript that details how fibroblast contamination was removed as previously described. The text within quotations was added for clarity per the reviewer’s comment.

Primary Melanocyte Culture & Immunofluorescence

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Primary melanocytes were isolated from P3 Tln1+/+ and Tln1E1770A/E1770A neonatal mice as previously described. Primary cultures were treated with 150 µM G418 for 4 days on, 3 days off for 6 weeks prior to use in experiments as “previously described in order to remove any” fibroblast contamination (Sviderskaya et al., 1997; Woodham et al., 2017). “This procedure was validated with Dct antibody staining before experiments were conducted for this manuscript.”

Second decision letter

MS ID#: DEVELOP/2019/184234

MS TITLE: Precise Coordination of Cell-ECM Adhesion is Essential for Efficient Melanoblast Migration During Development

AUTHORS: Amanda Haage, Kelsey Wagner, Wenjun Deng, Bhavya Venkatesh, Caitlin Mitchell, Katharine Goodwin, Aaron Bogutz, Louis Lefebvre, Catherine D Van Raamsdonk, and Guy Tanentzapf
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The revised manuscript provides additional evidence that regulation of talin autoinhibition is essential for efficient melanoblast migration in the mouse embryo. The new data analysis of protrusion number, length, and persistence gives a more comprehensive characterization of the phenotype when talin's autoinhibitory function is perturbed.

Comments for the author

The authors have sufficiently addressed the major concerns of the reviewers.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all of my comments and included both new experiments and new explanation where requested. I have no further requests for revision. This is a very nicely presented study and adds to our knowledge about how melanoblasts use their cytoskeleton and adhesion machinery to migrate to populate the skin and hair follicles.

Comments for the author

I have no further suggestions for revision.

Reviewer 3

Advance summary and potential significance to field

As stated in my initial review, I find this an interesting study about the regulation of integrin based adhesion by the auto-inhibition of its intracellular adaptor talin to effectively control the migration of neural crest derived melanoblasts in mice.

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This revised version has spent more effort on quantifying the cellular phenotypes of the talin auto-inhibition mutant, making the conclusions stronger that talin auto-inhibition is critical to control melanoblast cell shape, number, strength and dynamics of focal adhesions and finally their migration speed.

Comments for the author

All points raised have been successfully addressed in this revised version.
In summary, this is a carefully controlled study interesting to the integrin and developmental biology community.

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