Dia1 Coordinates Differentiation and Cell Sorting in a Stratified Epithelium

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February 11, 2021

Re: JCB manuscript #202101008

Dr. Margaret Gardel
University of Chicago
929 E 57th St GCIS E233
Chicago, IL 60605

Dear Dr. Gardel,

Thank you for submitting your manuscript entitled "Dia1 Coordinates Differentiation and Cell Sorting in a Stratified Epithelium". The manuscript has been evaluated by expert reviewers, whose reports are appended below. After an assessment of the reviewer feedback, our editorial decision is against publication of your manuscript in its current form.

As you will see, the reviewers found the studies of Dia1's role in differentiation and epithelium organization interesting. However, they also felt that the data leaves many questions open, in particular as to whether and how levels of Dia1 affect crowding in the basal keratinocyte layer and how this relates to its role in adhesion. They raised questions that undermine your model and would require further characterizations of Dia1-mediated processes. Although your manuscript is intriguing, we feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. Under the circumstances, if you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, we would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. While we do not feel that you would need to delve more deeply into the RNA-seq as suggested by Reviewer #1, all the other reviewer points would need to be addressed thoroughly for reconsideration at JCB. 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. We would be happy to discuss a revision strategy in the form of a detailed point-by-point response to the reviewer reports, if that is helpful.

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.

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

Sincerely,

Elaine Fuchs, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

The manuscript by Harmon et al. explores the role of Dia1 in epidermal organotypic cultures. Dia1 is particularly interesting since its distribution is restricted to the basal, proliferative layer of the epidermis, and its function is poorly understood.
The authors show that Dia1 depletion alters tissue morphogenesis, proliferation, and differentiation. The authors also describe defects in cell sorting and YAP/PRC activity.
While the study is potentially fascinating and important, many essential processes were poorly characterized (e.g., cell adhesion, actomyosin cytoskeleton, epidermal differentiation). Therefore, the interpretation of results is not simple. It is also unclear to what extent cell sorting, YAP, and PRC activities contribute to the complex phenotype.

Major point:
1. Dia1 is an important actin-binding protein; however, the authors barely characterized its effect on the keratinocyte actomyosin cytoskeleton. Will Dia1 depletion alter actin distribution? dynamics? levels? G/F actin ratio? What about Myosin II distribution and phosphorylation? These questions are essential since the actomyosin cytoskeleton plays a crucial role in cell sorting and epidermal differentiation.

2. Dia1 plays a role in cell adhesion (e.g., Acharya et al. 2017, Carramusa et al. 2007), a major regulator of epidermal biology. However, the authors barely characterized how Dia1 depletion affects cell-cell and cell-ECM adhesions. The latter is particularly interesting since the authors show that Dia1 localizes to the basal part of the cells where integrins function.
3. The role of Dia1 in epidermal differentiation requires additional characterization. Can the authors detect markers of the granular layer in the organotypic cultures? Will the cornified layer develop properly? What about basal layer keratins (keratin 5/14)? Stress keratins (keratin 6/16)? Moreover, on top of the hyper-proliferation, will Dia1 depletion induces cell death or stress?
4. The RNAseq experiments highlight the link between Dia1 and YAP and PRC in cultured cells. Unfortunately, the authors did not follow up on these interesting results (e.g., staining for YAP and its targets in the 2D and organotypic cultures). Moreover, can YAP/PRC manipulation rescue Dia1 differentiation and proliferation defects (see Zhou et al. 2013)?
5. In 2D Dia1 KD cells exhibit hypo proliferation (Fig. S4), while Dia1 KD exhibits hyper-proliferation in organotypic cultures (Fig. 2). Please explain the discrepancy.
6. Cell sorting experiments are fascinating. However, they further highlight the need to fully understand how Dia1 affects keratinocyte actomyosin cytoskeleton and adhesion, without which interpretation is problematic. Moreover, it is unclear to what extent defect in cell sorting contributes to the Dia1 KD phenotype in organotypic cultures.
7. Regarding experiments with dividing cells: It is well known that keratinocyte division involves mitotic rounding and spindle orientation. These processes are guided by cell polarity and strongly involve the actin cytoskeleton (Luxenburg et al., 2011; Williams et al., 2011; Williams et al., 2014). Does Dia1 depletion affect these processes?

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

In this manuscript the authors address a fundamental question in epithelial biology, how an epithelium maintains morphological and functionally distinct compartments and how individual cells sort to enable movement into a different compartment. Combining 3D and 2D keratinocyte cell culture models, the basic cell block of the multilayered stratified epidermis, the authors aim at addressing these questions and provide some evidence that the actin nucleator Dia1 regulates crowding-induced differentiation and regulates intercellular adhesion/protrusion in basal proliferative keratinocytes important for positioning cells after cell division. Their data suggest a model in which differential levels of the formin Dia1, position and sort out Dia1 high from low-Dia1 cells such that the later end up suprabasally to couple cell division with differentiation.

Although this paper has many potentially interesting observations, at present it has not rigorously tested all of the conclusions made and questions also remain on how some of the observations are connected. In addition, the paper suffers from insufficient reproducibility and the majority of the experiment seems to be based on N=2 independent experiments or even samples (RNA seq), resulting in inappropriate use of statistics and mixing technical and biological replicates, which limits the significance of the data at this point.

In principle the following major points should be addressed next to a range of specific experimental points.

1. Does Dia1 show differential levels in the control cells? The data will be considerably strengthened if the authors could show that downregulation or reorganization of Dia1 in individual cells is indeed an early event before cells delaminate/ differentiate, e.g. by staining sections of the 3d organotypic and/or (embryonic, as more divisions) skin. Their data would predict/suggest that reduced levels of Dia1 would occur in cells neighboring dividing cells.

2. The crowding data needs strengthening
First, these data lacks an important control i.e. subconfluent cells that are released after stretch. This control is essential as it cannot be ruled out that release of tension induces differentiation (similar to how stretching promotes stemness), instead of crowding, especially as Dia1 is an actin regulator and nuclear actin controls these pathways.
The authors in Figure 3 show a cherry-picked list of some individual genes, but it is surprising that the genes that were used to assess changes in differentiation in Figure 2 (i.e. K14, K10, K1, Involcrin) are absent in this list. Does crowding affects expression of these genes?
Most importantly, is crowding sufficient to overcome the 3D differentiation/positioning phenotype (non-differentiation Dia1-KD cells delaminate prematurely), e.g. by starting off with more cells?

3. At present the data in 2D and 3D show an apparent conflict; whereas Dia1-KD cells are overproliferating in 3D and move up despite being not crowded basally (Figure1 and 2), in 2D the cells are less proliferative (and less dense thus less crowded) actually results in strongly impaired delamination and stratification (Figure4). Both of which done with one cell type, not mixing of control and Diakd cells. How do the authors explain this? Only under conditions of mixing Dia1 cells preferentially delaminate/end up suprabasally in 2D conditions. As these are all short term assays, does mixing of Dia1-KD and control cells in 3D using different ratios (1;10 and vice versa to examine role of crowding as well), alter sorting behavior under these more physiologically relevant conditions?

4. It remains unclear how the experiments on crowding and the inability to form stable cell-cell adhesions are linked. The authors state that differentiation is later than changes in cell-cell adhesion, but they have no data to suggest either way, and whether

differentiation comes before upward movement is actually a huge question in the field that is not so easy to answer (see e.g. Miroshnikova et al., 2018; , as initial differentiation and cell-cell rearrangements are hard to separate. See also point 3, if crowding is sufficient to rescue sorting behavior and stratification in 2D then differentiation may come before cell-cell rearrangement. Dia1 deficient cells seem to be impaired not in their ability to move up (Fig.1, Fig.6F, even if they also stratify less which seems an apparent contradiction), but instead in their ability to move underneath dividing cells. Does crowding rescue this? or the impaired ability to stratify when switched to a differentiation medium?

5. The data shows indirect evidence that differences in the ability to establish intracellular traction forces is impaired in the Dia1-KD cells. But, this would be strengthened with either AFM or traction force data to show that intercellular tension/traction forces are altered.

6. The model presented in figure 7 is detailed on how Yap regulates plycomb an neuronal fate, however, this manuscript really did not examine this experimentally and these signatures only came up in the RNA seq, and should be removed from the model.

7. Specific Comments with respect to reproducibility and statistics:

Figure1:
All experiments and quantification are based on pooled data from two experiments. At least three experimental repeats/organotypic cultures are required and the mean values of each experiment have to be shown in the graphs to show repeatability. Epidermal organotypic cultures can vary a lot and thus the phenotypical consistency for ctr and KD needs to be shown. To test for significance instead of using T-test on pooled data points, the Test needs to be applied to the means of the experimental repeats. If N<5 a non-parametric T-test e.g. Mann-Whitney has to be used.

Figure2:
2b,d same comment on the statistics as in Figure1
2e,f Technical replicates of qPCRs cannot be used for statistical analysis. Only the means of the technical replicates per independent experiment or biological replicate can be used to test for significance. Also here, if <5 mean values are compared Mann-Whitney or similar has to be applied.

Figure3
3a It is not clear which statistical criteria have been used to calculate FDR values. The number of experiments has to be increased to perform either a parametric or non-parametric test or the individual fold regulation for each experiment should be shown without a significance value, as this is overstating the actual statistical significance
3b it is not clear what each dot represents. Perhaps each dot is a gene, but the graph lacks any information on the variance.
3d,e also here T-test cannot be used on just two samples, see comments above

Figure4
4b,c the means and error shown here have to be calculated based on the single means per experiments, not the mean of the pooled slides that were counted.
4e it is not clear if the percentages shown here are from one experiment or means.

Figure5
5b means of each experiment should be shown
5d-f it is not stated if the aggregates shown here are representative examples (of how many) or single observations

Minor comments:

1. It is unclear throughout the manuscript whether shRNA knockdown is used or CRISPR lines (which is also referred to as knockdown). How many clones were compared for shRNA? Or was the transfection done each time looking at a pool of cells? Was the Dia1-rescue generated using shRNA or CRISPR line?

2. Is the difference in nuclear orientation (Figure1) also obvious in 2D or a consequence of more tissue like organization? It is not clear if the nuclear aspect ratio were done on sections or 3D measurements, as using sections could potentially skew data. Is nuclear volume affected by the loss of Dia1 (as it seems in Figure1F).

3. The authors state that the aspect ratio sharply increases upon differentiation but that seems somewhat of an overstatement as this only seems to happen in the most upper suprabasal layers but actually initially decreases (0-0.8 in Figure1D) going from basal to suprabasal, the point on which the authors focus. It would be helpful in 1B to have a more precise estimate where on the scale from 0-1.0 on the x-axis in Figure1D-E is.

4. The quality of the PCNA data (Figure2C) is not great. It is not clear what is counted as PCNA positive as PCNA positive cells are also present in the suprabasal layers of the control cells. PCNA is not considered the most sensitive marker for examining proliferation, showing these changes using ki67 or Brdu incorporation might be better.

5. It would be nice to show the 18% prestretch cell density as well in Figure3C-D and not just stating that in the text.

6. Can the authors comment on why 35% of the Ctr cells lacked suprabasal partners in Figure4E?

7. The authors claim that heterotypic interfacial tension guides Dia1KD cells to sort to suprabasal layers. But this at this point is only speculative and should be stated in discussion rather then in the main text.

8. As actin is coupled to AJs at cell-cell contact sites, how do these junction looks like in Dia1-KD cells (Figure5A).

9. For clarity, do I understand correctlty that in the presence of the rock inhibitior is it a single flat? layer of aggregrated cells is formed in the hanging drop assay (Figure5F-ROCK inhibitor)?

10. The authors state based on the sorting experiment in Figure5f that Rock activity drives Dia1 dependent sorting and stratification. This statement is based on an assay that very likely does not represent physiological stratification. The results thus suggest but do proof that this is the case and the conclusion should be adapted.

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

In this manuscript, Robert Harmon et al. document a novel role for the canonical formin Dia1 in regulating epidermal compartmentalization through cell sorting effects and epidermal stratification. The findings are of interest to the field; however, additional clarifications and experiments would be helpful to substantiate their claims, as outlined below:

Major comments:

1. Functional roles for Dia1 in the compartmentalization of the basal epidermal layer. Is this effect only due to Dia1's function in cell-cell addition and actin contractility?

An alternative explanation is the role of Dia1 in Focal Adhesion (FA) assembly and cell polarity maintenance. FA's mechanosensitivity is also involved in maintaining the basal layer, cell density, basal layer organization, and differentiation defects.

Indeed, the data shown in Fig. S1D is intriguing. Dia1KD cells seem to invade the collagen layer, indicating an increased cell migration or invasion. Cellular crowding can also inhibit those effects.

2. In the same line of ideas, the authors document that the loss of Dia1 leads to a reduction of the basal layer density and attribute the effect to the loss of cell-cell adhesion. Could that also be related to an increase in cell spreading, as observed in Fig 4F?

3. The loss of Dia1 results in increased basal cell proliferation and hyperplasia (Fig 2C-D). How do the authors reconcile those findings to the ones shown in Fig. 4B, where the cells populate the culture at a reduced rate and suggest that it is possibly due to reductions in cell proliferation?

4. Roles of Dia1 in maintaining the basal cell identity. The authors conducted rescue experiments re-expressing the full-length Dia molecule in Dia1KD cells and conducted transcriptome analyses. However, the associated genes found in their transcriptomic analyses are related to keratinization, cornification, and genes related to differentiation, e.g., Notch. Fig. 3A, B. Where cells exposed to a calcium switch before the analyses?

5. Dia1 in differential cell sorting. Under normal conditions, are there any differences in Dia1expression levels associated with compartmentalized areas in the epidermis? How is Dia1 downregulated in the basal layer? Wouldn't the Dia1 negative cell be already committed to differentiation and later undergo the proposed delamination model once crowding by a neighboring cell division? Discuss these scenarios.

We thank the three reviewers for carefully reading our manuscript and providing thoughtful suggestions for its improvement. In response, we have performed new experiments and analysis, resulting in the inclusion of 5 new figure panels in the main text and 9 new supplemental figures. We have also included 5 figures in this review response that we felt didn't add to the main conclusions of the manuscript.

We now include data with improved characterization of cell-cell adhesion and included an experiment to dissect out the role of crowding and differential cell-cell adhesion. These data all support our idea that it is the differential cell-cell adhesion, rather than changes to cell crowding the support the extrusion of Dial1 KD cells.

We note that the editor did not feel necessary to delve further into the RNA-seq data, a suggestion from Reviewer 1. We hope the reviewers now find our work suitable for publication in JCB.

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

The manuscript by Harmon et al. explores the role of Dia1 in epidermal organotypic cultures. Dia1 is particularly interesting since its distribution is restricted to the basal, proliferative layer of the epidermis, and its function is poorly understood.
The authors show that Dia1 depletion alters tissue morphogenesis, proliferation, and differentiation. The authors also describe defects in cell sorting and YAP/PRC activity.
While the study is potentially fascinating and important, many essential processes were poorly characterized (e.g., cell adhesion, actomyosin cytoskeleton, epidermal differentiation).
Therefore, the interpretation of results is not simple. It is also unclear to what extent cell sorting, YAP, and PRC activities contribute to the complex phenotype.

Major point:
1. Dia1 is an important actin-binding protein; however, the authors barely characterized its effect on the keratinocyte actomyosin cytoskeleton. Will Dia1 depletion alter actin distribution? dynamics? levels? G/F actin ratio? What about Myosin II distribution and phosphorylation? These questions are essential since the actomyosin cytoskeleton plays a crucial role in cell sorting and epidermal differentiation.

We find the strongest phenotype of Dia1 depletion to be on the formation of perijunctional actomyosin cables that form proximal to the cell-cell junction on the apical cell surface 3-4 hours post plating. For context, it is believed that this population compacts into peripheral bundles as adhesions mature and, in terms of localization, becomes nearly indistinguishable from the junctional actin pool (Rajakyla et al., 2020). We now include supplemental immunostaining experiments to corroborate the live cell actin data provided in Figure 5C.

We now include a new supplementary figure, Fig. S12, that characterizes the actomyosin cytoskeleton at this time point. In Fig. S12A, western blotting reveals a 40% decrease in phospho-MLC (pMLC) levels in the Dia1KD cultures at 4hrs post-plating. Prior characterizations of actin in keratinocytes suggest pMLC localizes to perijunctional bundles

(Vaezi et al., 2002). In Fig. S12B-C, we show immunofluorescence images of phalloidin (F-actin) and pMLC at the apical, middle and basal layers in Control and Dia1KD cells The most notable distinguishing figure is the absence of pMLC in the perijunctional bundles (section ii).

To quantify these observations, we performed line scans of the apical junction plane as well as mean intensity measurements of pMLC and actin at specified positions in the cell shown in Fig. S12D-G. These supported a role for Dia1 in pMLC accumulation at the perijunctional region. Note that, in Dia1KD cultures, apical junctions do not always extend to tricellular junctions, which exist at an aberrantly basal plane. This creates a discontinuous staining pattern. As such, only the region marked by junctional actin was analyzed to avoid analyzing empty space. Surprisingly, at 4hrs, with this caveat and those associated with 3D imaging noted, overall actin intensity did not appear diminished in Dia1KD cultures, and appeared somewhat increased. For context, Acharya et al (Acharya et al., 2017) noted an effect on both intensity and organization of actin cytoskeleton, but imaged at a much later timepoint and in a different cell type. We conclude that Dia1 supports the development of actomyosin cabiles on cell apices. Whether Dia1 directly polymerizes the perijunctional actin pool or is involved in the production of a precursor population remains unanswered. This is a difficult task even in simple single cell systems given the interdependence of stress fiber pools.

Control cells at this timepoint possess a relatively disorganized actin network on their basal surface (see Reviewer Figure 1). Discernible differences in the Dia1KD cells were not detected, and as such we did not further investigate this region. The lateral domain of the cortex, midway between the bottom and top of the cell accumulates actin at this timepoint in both control and Dia1KD cells (Fig. S12). However, it is somewhat difficult to compare the two conditions as the Dia1KD cells experience delays in vertically extending the lateral surface (see cross sections Reviewer Figure 1).

At later timepoints (24hrs), Dia1KD cells do succeed at extending the lateral adhesive domain vertically to more closely match the height of the cell (Reviewer Figure 2). Under these conditions, both controls and Dia1KD cells accumulate actin on the lateral domain. Note that the apical architecture becomes dominated in both conditions by the appearance of a medioapical actin network which coincides temporally with the cultures beginning to stratify. This medioapical network contains weak phospho-MLC staining which is indistinguishable between the CTL and Dia1KD conditions. Thus, we conclude that Dia1 primarily functions in the early stages of keratinocyte adhesion development, specifically, in the generation of phospho-myosin decorated perijunctional actin cables.

2. Dia1 plays a role in cell adhesion (e.g., Acharya et al. 2017, Carramusa et al. 2007), a major regulator of epidermal biology. However, the authors barely characterized how Dia1 depletion affects cell-cell and cell-ECM adhesions. The latter is particularly interesting since the authors show that Dia1 localizes to the basal part of the cells where integrins function.

Thank you for this suggestion. We have now conducted additional immunofluorescence staining of E-cadherin and vinculin and show this data in Fig. S13.

Part of the reason we did not focus on cell-ECM adhesions is that we did not notice a strong phenotype. Notably, anecdotally both CTL and Dia1KD cells adhered rapidly to collagen gels (<30min) when we prepare the samples. Moreover, the cell-ECM adhesions in control cells in our cultures, as visualized by paxillin, are barely discernible (Reviewer Figure 3) so differences via immunostaining between the Control and Dia1KD cells are not readily observed. Thus, we did not observe a strong adhesion phenotype warranting further investigation.

In terms of cell-cell adhesion, we have observed the starkest phenotype at the early stages of cell-cell adhesion assembly, 4hrs post plating. After this time, control cells have already constructed a lateral adhesive domain that extends nearly as high as the cell roof (Fig. 7A). In contrast, Dia1KD cells have a restricted lateral adhesive domain (Fig. 7A). We now present images that include E-cadherin immunofluorescence in Fig. S13. E-cadherin accumulates in a polarized fashion along the lateral surface, favoring the apically skewed localization of classic adherens junctions (Fig. S13A,C,E). This places the adhesions in close proximity to perijunctional actomyosin cables found exclusively in control cells.

We speculate that this arrangement might place E-cadherin-based junctions under tension at this time point in adhesion development. We have now tested this by performing immunofluorescence of vinculin, an adaptor protein recruited specifically to junctions under tension(Seddiki et al., 2018), in a new Fig. S13. Indeed, in Control cells, there is partial co-localization of vinculin with E-cadherin on the apical surface (Fig. S13A,D,F).

In contrast, the E-cadherin staining of apical junctions in Dia1KD cultures is discontinuous because the tricellular junctions lie in an aberrantly low plane (Fig. S13). When limited to analyzing the most apical patches of E-cadherin we did not detect an overall drop in mean E-cadherin intensity in Dia1KD cultures despite the altered cell morphology. At 24hrs, E-cadherin does appear somewhat reduced, in line with earlier publications (Acharya et al., 2017; Carramusa et al., 2007) (see Reviewer Figure 4). Nonetheless, at 4hrs, vinculin does not co-localize with E-cadherin in the apical junctions of Dia1KD cells.

This data suggests that Dia1 is important role in the rapid assembly and maturation of apical junctions during the initial phases of cell-cell adhesion.

3. The role of Dia1 in epidermal differentiation requires additional characterization. Can the authors detect markers of the granular layer in the organotypic cultures? Will the cornified layer develop properly? What about basal layer keratins (keratin 5/14)? Stress keratins (keratin 6/16)? Moreover, on top of the hyper-proliferation, will Dia1 depletion induces cell death or stress?

Though HaCaTs do provide certain technical advantages as a system which reliably stratifies and expresses certain differentiation markers, they do take a significantly longer time to form proper granular or cornified layers than cultures produced from primary tissue (Shoop et al 1999). As we did not seek to study the most terminal aspects of differentiation, we opted for shorter incubation times and will clarify the limitations of HaCaT cultures more clearly in the text.

That said, qPCR data showed clear signatures in control HaCaT cultures of early differentiation markers (e.g. KRT1 and IVL). Further, we found this signature was disrupted by Dia1 depletion (Fig. 2E) and rescued by its re-expression (Fig. 2F).

The RNA-seq data also underscored the role of Dia1 in epidermal differentiation, where there is enrichment in gene sets that serve as markers of cornification, keratinization, keratinocyte differentiation, epidermal cell differentiation and epidermis development (Fig. 3A). Keratin 5 and Keratin14 were included in the GO:0008544 and were enriched by 35% and 40%, respectively, in rescue cells. Keratin 6B and Kerain16 were included in GO:0031424 and were enriched by 350% and 100%, respectively. The idea of an effect on keratins associated with stress is very interesting as you could imagine Dia1 being important during reestablishment of a wounded epithelium given its dysregulation in diabetic wounds

We have not seen any indications that Dia1 depletion induces cell death and did not pursue further investigation.

4. The RNAseq experiments highlight the link between Dia1 and YAP and PRC in cultured cells. Unfortunately, the authors did not follow up on these interesting results (e.g., staining for YAP and its targets in the 2D and organotypic cultures). Moreover, can YAP/PRC manipulation rescue Dia1 differentiation and proliferation defects (see Zhou et al. 2013)?

Thanks for this intriguing suggestion! We have, indeed, seen some preliminary evidence that YAP/PRC manipulation can rescue Dia1KD phenotypes but feel this is beyond the scope of this current body of work. We agree this would be exceptionally interesting to pursue in future studies.

5. In 2D Dia1 KD cells exhibit hypo proliferation (Fig. S4), while Dia1 KD exhibits hyper-proliferation in organotypic cultures (Fig. 2). Please explain the discrepancy.

Thank you for this question. In wild type conditions, the cell proliferation becomes suppressed in mature organotypic cultures. Compared to this, the initial proliferation rate is suppressed in Dia1KD cells (Fig. S8) but, more importantly, their proliferation is not suppressed during the long term maturation of 3D organotypic cultures.

Thus, we believe the discrepancy in these data arises from the time scale of the cultures. The 2D experiments are short term, lasting no more than 4 days, and probe the proliferation in immature monolayers. The 3D cultures have been grown for 1-2 weeks, and probe the proliferation in mature organotypic models. We have now clarified this better in the text.

6. Cell sorting experiments are fascinating. However, they further highlight the need to fully understand how Dia1 affects keratinocyte actomyosin cytoskeleton and adhesion, without which interpretation is problematic. Moreover, it is unclear to what extent defect in cell sorting contributes to the Dia1 KD phenotype in organotypic cultures.

We have included several new pieces of data to address this important question. We now include two new supplementary figures, Fig. S12 and S13 to examine changes in the actomyosin cytoskeleton and cell-cell adhesion.

Further, we now show new data in Figure 5D-H of contact angle measurements to assess contact angle measurements between cell pairs both within monolayers as they develop on collagen gels, to assess relative changes of apical vs. basal tension, as well as from cell doublets in suspension, to isolate cell-cell adhesion effects. These measurements allow for the relative comparison of adhesive and cortical forces imparted upon different cellular domains. Dia1 expression specifically favored development of tension across the apical surface. As contractile boundaries are known to prevent cell movement (Monier et al., 2010), this is consistent with the hypothesis that Dia1 expression would prevent premature loss of cells to the suprabasal compartment.

Likewise, contact angle measurements between cell pairs show similar values between homotypic cell pairs (Dia1+/Dia1+; Dia1-/Dia1-) (Figure 5F-H). By contrast, heterotypic cell doublets demonstrated a failure of Dia1+/Dia1- pairs to extend an adhesive interface (Figure 5F-H). Together, these data suggest that during the initial states of cell-cell adhesion, cells with diminished Dia1 expression may be forced out of the basal layer preferentially. New data presented in Fig S10, examining the positioning of Dia1KD cells diluted 50:1 amongst CTL cells, confirms this preference and provides a more physiological correlate to the cell sorting experiments (now located in Figure 6).

7. Regarding experiments with dividing cells: It is well known that keratinocyte division involves mitotic rounding and spindle orientation. These processes are guided by cell polarity and strongly involve the actin cytoskeleton (Luxenburg et al., 2011; Williams et al., 2011; Williams et al., 2014). Does Dia1 depletion affect these processes?

We assessed symmetric and asymmetric division in live H2B labeled cultures and found that CTL cells were more likely than Dia1KD cultures to divide asymmetrically after 24hrs in culture (~24% vs ~6%) (Fig. S11). Box et al (Box et al., 2019) would argue that this result is consistent with the Dia1KD monolayers being ~20% less dense than CTL cells. For comparison, density values in Fig. S11 suggest an average cell area of ~140um^2 for CTL cells, close to the value determined by Box et al for wild-type basal keratinocytes in embryonic mice. Dia1KD cells averaged ~170um^2. This argues against basal density defects, at least during the initial development of a suprabasal compartment, stemming from asymmetric division at inappropriately low cell densities.

No obvious polarity defects have been observed via Par3 staining and/or centrosome positioning.

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

In this manuscript the authors address a fundamental question in epithelial biology, how an epithelium maintains morphological and functionally distinct compartments and how individual cells sort to enable movement into a different compartment. Combining 3D and 2D keratinocyte cell culture models, the basic cell block of the multilayered stratified epidermis, the authors aim at addressing these questions and provide some evidence that the actin nucleator Dia1 regulates crowding-induced differentiation and regulates intercellular adhesion/protrusion in basal proliferative keratinocytes important for positioning cells after cell division. Their data suggest a model in which differential levels of the formin Dia1, position and sort out Dia1 high from low-Dia1 cells such that the later end up suprabasally to couple cell division with differentiation.

Although this paper has many potentially interesting observations, at present it has not rigorously tested all of the conclusions made and questions also remain on how some of the observations are connected. In addition, the paper suffers from insufficient reproducibility and the majority of the experiment seems to be based on N=2 independent experiments or even samples (RNA seq), resulting in inappropriate use of statistics and mixing technical and biological replicates, which limits the significance of the data at this point.

In principle the following major points should be addressed next to a range of specific experimental points.

1. Does Dia1 show differential levels in the control cells? The data will be considerably strengthened if the authors could show that downregulation or reorganization of Dia1 in individual cells is indeed an early event before cells delaminate/ differentiate, e.g. by staining sections of the 3d organotypic and/or (embryonic, as more divisions) skin. Their data would predict/suggest that reduced levels of Dia1 would occur in cells neighboring dividing cells.

This is a fascinating question. Unfortunately, little is known about the timing or mechanisms by which Dia1 expression is modulated at the transcriptional and post-transcriptional level. There is some evidence that miR-198 targets Dia1 (Sundaram et al., 2017) . Though there is variation in staining intensity, we were unable to clearly identify Dia1-negative cells within that compartment in fixed organotypic cultures and assume that such cells might be rapidly expelled to the suprabasal layers. For comparison, how and when expression of classic differentiation markers, like KRT10, is initiated in basal cells remains poorly understood and is not always clear from histology. Addressing this question will require advances in genetic and imaging tools developed by groups like the Greco and Lechler labs (Cockburn et al., 2021; Muroyama and Lechler, 2017). Unfortunately, we do not have the tools available to address this question in this current study.

2. The crowding data needs strengthening. First, these data lacks an important control i.e. subconfluent cells that are released after stretch. This control is essential as it cannot be ruled out that release of tension induces differentiation (similar to how stretching promotes stemness), instead of crowding, especially as Dia1 is an actin regulator and nuclear actin controls these pathways.

We thank you for this important suggestion, we have now performed these experiments. In Fig. S7, we now show qPCR data of two differentiation markers CERS3 and KRT1 on sub-confluent cultures that are subjected to enforced crowding. Crowding does not drive increased expression of these differentiation markers in sub-confluent monolayers.

The authors in Figure 3 show a cherry-picked list of some individual genes, but it is surprising that the genes that were used to assess changes in differentiation in Figure 2 (i.e. K14, K10, K1, Involcrin) are absent in this list. Does crowding affects expression of these genes?
Differentiation markers have different kinetics after the induction of differentiation and those, in turn, differ between methods of induction. Numerous studies (Mishra et al., 2017), (Ichikawa et al., 2008), (Liebig et al., 2009),(Toufighi et al., 2015)). Totaro et al (Totaro et al., 2017), demonstrate that induction of IVL by restricting cell area lags well behind the YAP signaling events (targets of which are noted in Fig.3) which are thought to give rise to that induction. The data in Figure 2 was taken 48 hours after a Ca-switch experiment whereas the data in Figure 3 was taken 5 hours after enforced crowding. To explore the idea that crowding can affect expression of K1, we now show qPCR data from 9 hours after enforced crowding in Fig. S7.

Most importantly, is crowding sufficient to overcome the 3D differentiation/positioning phenotype (non-differentiation Dia1-KD cells delaminate prematurely), e.g. by starting off with more cells?
This is a fantastic question. In our experiments, the initial plating density is saturated and cannot drive the 3D differentiation phenotype.

3. At present the data in 2D and 3D show an apparent conflict; whereas Dia1-KD cells are overproliferating in 3D and move up despite being not crowded basally (Figure1 and 2), in 2D the cells are less proliferative (and less dense thus less crowded) actually results in strongly impaired delamination and stratification (Figure4). Both of which done with one cell type, not mixing of control and Diakd cells. How do the authors explain this?
Thank you for this question. In wild type conditions, the cell proliferation becomes suppressed in mature organotypic cultures. Compared to this, we believe the initial proliferation rate to be suppressed in Dia1KD cells (Fig. S8) but, more importantly, their proliferation is not suppressed during the long term maturation of 3D organotypic cultures.

Thus, we believe the discrepancy in these data arises from the time scale of the cultures. The 2D experiments are short term, lasting no more than 4 days, and probe the proliferation in immature monolayers. The 3D cultures have been grown for 1-2 weeks, and probe the proliferation in mature organotypic models. We have now clarified this better in the text.

Only under conditions of mixing Dia1 cells preferentially delaminate/end up suprabasally in 2D conditions. As these are all short term assays, does mixing of Dia1-KD and control cells in 3D using different ratios (1;10 and vice versa to examine role of crowding as well), alter sorting behavior under these more physiologically relevant conditions?

We thank the reviewer for suggesting that we explore the positioning of Dia1KD cells in the context of a crowded monolayer during the formation of a suprabasal compartment. We now show this data in Fig. S10 where we've probed the position of cells formed in mixed cultures comprised of CTL and Dia1-KD cells at a 50:1 ratio (Fig. S10A). The cell density of these cultures is similar to control cultures. We find that the Dia1KD cells preferentially are found in the suprabasal compartment and this positioning is ROCK-dependent (Supplementary Figure 10B-C).

4. It remains unclear how the experiments on crowding and the inability to form stable cell-cell adhesions are linked. The authors state that differentiation is later than changes in cell-cell adhesion, but they have no data to suggest either way, and whether differentiation comes before upward movement is actually a huge question in the field that is not so easy to answer (see e.g. Miroshnikova et al., 2018; , as initial differentiation and cell-cell rearrangements are hard to separate.

We agree that the coordination between differentiation and upward movement is an outstanding question in the field that we have not addressed here. We have now reviewed the text carefully to ensure there are no statements about the relative timing of these processes.

See also point 3, if crowding is sufficient to rescue sorting behavior and stratification in 2D then differentiation may come before cell-cell rearrangement. Dia1 deficient cells seem to be impaired not in their ability to move up (Fig.1, Fig.6F, even if they also stratify less which seems an apparent contradiction), but instead in their ability to move underneath dividing cells. Does crowding rescue this? or the impaired ability to stratify when switched to a differentiation medium?

Thank you for this suggestion. In Fig. S10 we now show data of the movement of Dia1 KD cells that are sparsely embedded into a monolayer formed of control cells. The density of these cells is similar to control monolayers. In this experiment (which is done in differentiation medium), Dia1 KD cells preferentially are found in the suprabasal layer. This is consistent with our picture that Dia1 KD are impaired in their ability to remain in the basal layer.

5. The data shows indirect evidence that differences in the ability to establish intracellular traction forces is impaired in the Dia1-KD cells. But, this would be strengthened with either AFM or traction force data to show that intercellular tension/traction forces are altered.

Thank you for this suggestion. We want to be clear in distinguishing between kinetics of adhesion assembly and the adhesion forces at these various time points. Fig. 7 shows that Dia1-KD cells have impaired cell-cell adhesion assembly kinetics. At a time point immediately after a full cell-cell contact is achieved, we show in Fig. 5A-C and Fig. S12 that the Dia1KD cells are impaired in cortical acto-myosin assembly and don't distort the underlying collagen gel, suggestive of reduced cell-cell force along the lateral membrane.

We did perform traction force microscopy of cells on collagen and E-cadherin substrates to isolate force generation in the presence of integrin and E-cadherin-mediated adhesion and didn't see a significant difference (Reviewer Figure 5). This shows that the changes are not arising from steady state changes in cellular force generation in the absence of the epithelial context.

To explore this further, we have now performed cell-cell adhesion assays to probe the relative tension between cell-cell adhesion force and cortical tension in doublets of homotypic and heterotypic cell pairs. Intriguingly, we found that homotypic pairs formed either of Control or Dial1KD cells had a higher contact angle than the heterotypic pairs (Fig. 5). This indicates in homotypic cell pairs the relative tension of cell-cell adhesion to cortical tension is quite similar. Our data in Fig. 5A indicates that higher levels of lateral force in the CTL cells compared to Dia1KD cells. This then suggests that the reduced contact angle of heterotypic cell pairs arises from differences in lateral membrane tension and cortical tension between the Control and Dia1KD cells, indicating a reduced capability of Dia1KD cells to rapidly integrate within the monolayer.

6. The model presented in figure 7 is detailed on how Yap regulates plycomb an neuronal fate, however, this manuscript really did not examine this experimentally and these signatures only came up in the RNA seq, and should be removed from the model.

Thank you for this important suggestion and we have modified the figure (now Figure 8) and the manuscript. As these could be important points to follow up, we will retain but limit our discussion of these pathways.

7. Specific Comments with respect to reproducibility and statistics:

Figure1:
All experiments and quantification are based on pooled data from two experiments. At least three experimental repeats/organotypic cultures are required and the mean values of each experiment have to be shown in the graphs to show repeatability. Epidermal organotypic cultures can vary a lot and thus the phenotypical consistency for ctr and KD needs to be shown. To test for significance instead of using T-test on pooled data points, the Test needs to be applied to the means of the experimental repeats. If N<5 a non-parametric T-test e.g. Mann-Whitney has to be used.
We have now included an additional data set for Fig. 1C. In Fig. S1, we show two additional data sets for the data shown in Figure 1D+E.
We corroborated the density measured in Fig. 1G by measuring density differences in a fixed data set and obtained consistent measurements. This data is now shown in Fig. S3.

Figure2:
2b,d same comment on the statistics as in Figure1

For Fig. 2B, 2D we now show data for 3 experimental repeats. We now also plot this data in Fig. 2D using box plots to show the distribution for each individual experiment.

2e,f Technical replicates of qPCRs cannot be used for statistical analysis. Only the means of the technical replicates per independent experiment or biological replicate can be used to test for significance. Also here, if <5 mean values are compared Mann-Whitney or similar has to be applied.

We have now done n=3 independent replicates for Figure 2E and 2F per the reviewer suggestions. Our understanding is that a Whitney Mann test requires n>5 replicates. We have followed a protocol from a recent paper from the Schekman lab that used three replicates and compared with a t-test (Song et al., 2021)

Figure3
3a It is not clear which statistical criteria have been used to calculate FDR values. The number of experiments has to be increased to perform either a parametric or non-parametric test or the individual fold regulation for each experiment should be shown without a significance value, as this is overstating the actual statistical significance.

We have now removed this significance value from Fig. 3A.

3b it is not clear what each dot represents. Perhaps each dot is a gene, but the graph lacks any information on the variance.

In Fig. 3B, the dots are genes and the values are averages taken from duplicate experiments. This is now clearly stated in the figure legend.

3d,e also here T-test cannot be used on just two samples, see comments above
While each gene in and of itself may not prove statistically interesting, we believe the trends amongst, for example, a set of genes corresponding to skin disease are unlikely to be by chance and are worth reporting. In Fig. 3E what is being compared is the behavior of a subset composed of hundreds of genes compared to the behavior of all genes detected (>15000) or a random subset. A T-test would seem appropriate in comparing such distributions (Luo et al., 2009)

Figure4
4b,c the means and error shown here have to be calculated based on the single means per experiments, not the mean of the pooled slides that were counted.

Fig. 4B&C now shows unpooled data from a single experiment. We show this data from a second time course in Figure S8.

4e it is not clear if the percentages shown here are from one experiment or means.

Thank you for this suggestion. We have now reformatted this data as a table showing the data from 3 independent experiments.

Figure5
5b means of each experiment should be shown

Thank you for this suggestion. We have now modified Figure 5B so that the means of each experiment are shown. Also, note, that we have converted the collagen deformation data into an angle measurement to be more consistent with the additional contact angle data.

5d-f it is not stated if the aggregates shown here are representative examples (of how many) or single observations

Note, these have moved to Figure 6. These are representative examples of the aggregates used in calculating the separation index described in the text. The sample size is now indicated in the text.

Minor comments:
1. It is unclear throughout the manuscript whether shRNA knockdown is used or CRISPR lines (which is also referred to as knockdown). How many clones were compared for shRNA? Or was the transfection done each time looking at a pool of cells? Was the Dia1-rescue generated using shRNA or CRISPR line?

We apologize for this confusion. We have now thoroughly checked our naming conventions to be consistent.

CTL: Parental HaCaT line
Dia1KD: CRISPR-edited line derived from CTL
CTL-H2B: Derivative of CTL transduced with GFP-H2B lentivirus
Dia1KD-H2B: Derivative of Dia1KD transduced with GFP-H2B lentivirus
CTL-Scarlet: Derivative of CTL transduced with Scarlet lentivirus
Dia1KD-Scarlet: Derivative of Dia1KD transduced with Scarlet lentivirus
Dia1-Rescue: Derivative of Dia1KD transduced with Scarlet-tagged mDia1 lentivirus

shCTL: Parental HaCaTs transduced with a non-targetic pGIPZ shRNA lentivirus
shDia1: Parental HaCaTs transduced with a Dia1-targeting pGIPZ shRNA lentivirus

The shRNA chosen to knockdown Dia1 was previously published by Bovellan et al (2014). Lentivirus were used to infect parental HaCaTs and the cells were mass selected via puromycin to kill off uninfected cells and used for subsequent experiments. No cloning was done of the shRNA treated cells. The CRISPR line was used to generate rescue cells.

2. Is the difference in nuclear orientation (Figure1) also obvious in 2D or a consequence of more tissue like organization?

Differences in the nuclear shape are also seen in 2D cultures, as shown in Figure 4A. However, in Figure 1 we were primarily interested in characterizing the differences in a organotypic context.

It is not clear if the nuclear aspect ratio were done on sections or 3D measurements, as using sections could potentially skew data.

The basal cell density and nuclear aspect ratios were done on 3 independent sectioning experiments, which are now shown independently in Fig. 1C and Fig. S1

Is nuclear volume affected by the loss of Dia1 (as it seems in Figure1F).

This is an intriguing question that we have not explored in this current work.

3. The authors state that the aspect ratio sharply increases upon differentiation but that seems somewhat of an overstatement as this only seems to happen in the most upper suprabasal layers but actually initially decreases (0-0.8 in Figure1D) going from basal to suprabasal, the point on which the authors focus. It would be helpful in 1B to have a more precise estimate where on the scale from 0-1.0 on the x-axis in Figure1D-E is.

Thank you for pointing this out. We do not wish to conclude there are large differences in the nuclear aspect ratio between the cell types or as a function of vertical position. This is important to consider in our measurements of nuclear orientation, shown in Fig. 1E. We have now carefully gone through the text to ensure this is communicated clearly.
We have modified the cartoon in Fig. 1B to make this relative position scale more accurate and useful to compare with data shown in Fig. 1D and E.

4. The quality of the PCNA data (Figure2C) is not great. It is not clear what is counted as PCNA positive as PCNA positive cells are also present in the suprabasal layers of the control cells. PCNA is not considered the most sensitive marker for examining proliferation, showing these changes using ki67 or Brdu incorporation might be better.

PCNA staining has been widely used to examine proliferation in these types of organotypic cultures and our CTL cells match those previous reported in the literature (Akgul et al., 2005),(Yuan et al., 2020). Thus, it is possible to compare these data to the Dia1KD model tissues. We have now clarified how the data were quantified in the methods. In brief, nuclei were considered pcna positive if the intensity within nuclear masks exceeded 1.5x the mean signal obtained from CTL suprabasal nuclei between relative heights 0.7-0.8.

5. It would be nice to show the 18% prestretch cell density as well in Figure3C-D and not just stating that in the text.

We have now measured the changes in cell density with 18% pre-stretch and have put this relative increase in density in Fig. S6.

6. Can the authors comment on why 35% of the Ctr cells lacked suprabasal partners in Figure4E?

We believe this is an effect of heterotypic cultures. Since the Dia1KD cells migrate atop control neighbors, this alleviates the crowding pressure which would normally trigger control cells to stratify is reduced, leaving to a reduced number of cells with suprabasal partners.

7. The authors claim that heterotypic interfacial tension guides Dia1KD cells to sort to suprabasal layers. But this at this point is only speculative and should be stated in discussion rather then in the main text.

We now present data in Figure 5 on the interfacial tension to further bolster this idea. We also carefully review our language to ensure we are not overstating our claims.

8. As actin is coupled to AJs at cell-cell contact sites, how do these junction looks like in Dia1-KD cells (Figure5A).

We have now included supplemental data addressing actin organization during assembly of cell-cell contacts in Fig. S11. Please also see response to Reviewer 1 on page 2.

9. For clarity, do I understand correctlty that in the presence of the rock inhibitior is it a single flat? layer of aggregrated cells is formed in the hanging drop assay (Figure5F-ROCK inhibitor)?

This is correct. We have now clarified this in the text.

10. The authors state based on the sorting experiment in Figure5f that Rock activity drives Dia1 dependent sorting and stratification. This statement is based on an assay that very likely does not represent physiological stratification. The results thus suggest but do proof that this is the case and the conclusion should be adapted.

We have modified the language in the text to tone down this conclusion. We have also now included new data in Fig. S10 to further bolster this claim. This is data of the suprabasal positioning of both CTL and Dia1KD cells in a heterotypic culture formed with control:Dia1KD cells at a 50:1 ratio. Here, Dia1KD cells are preferentially driven to the suprabasal layer, despite being surrounding by control cells. We found that ROCK inhibition abrogates the preferential suprabasal positioning of the Dia1KD cells.

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

In this manuscript, Robert Harmon et al. document a novel role for the canonical formin Dia1 in regulating epidermal compartmentalization through cell sorting effects and epidermal stratification. The findings are of interest to the field; however, additional clarifications and experiments would be helpful to substantiate their claims, as outlined below:

Major comments:

1. Functional roles for Dia1 in the compartmentalization of the basal epidermal layer. Is this effect only due to Dia1's function in cell-cell addition and actin contractility?

An alternative explanation is the role of Dia1 in Focal Adhesion (FA) assembly and cell polarity maintenance. FA's mechanosensitivity is also involved in maintaining the basal layer, cell density, basal layer organization, and differentiation defects.

Indeed, the data shown in Fig. S2D is intriguing. Dia1KD cells seem to invade the collagen layer, indicating an increased cell migration or invasion. Cellular crowding can also inhibit those effects.

These are intriguing hypotheses and we have considered these.

First, we note that the stratification/compartmentalization of differentiated and undifferentiated cells can occur in suspension without an extracellular matrix (Watt, 1984). In our experiments, we found that both Control and Dia1KD cells rapidly attached to collagen gels suggesting both could adhere to the ECM. Further, immunostaining for focal adhesion markers paxillin and vinculin revealed only diffuse staining on the basal layer (Supplemental Figure S11, Reviewer Figure 3), suggesting small focal adhesion punctae that are typically less force-sensitive. All of these suggest a minimal role of focal adhesion mechanosignaling in the establishment of the basal layer.

Previous work has shown that Dia1KD in keratinocytes leads to reduced motility (Sundaram et al., 2013).

We suspect that the morphology observed in Fig. S2D of the Dia1KD cell tissue arises from mis-organization of tension, rather than an aberrant motility or invasion phenotype. However, this will require further investigator.

2. In the same line of ideas, the authors document that the loss of Dia1 leads to a reduction of the basal layer density and attribute the effect to the loss of cell-cell adhesion. Could that also be related to an increase in cell spreading, as observed in Fig 4F?

We agree with the reviewer that there are several ways to interpret changes in cell morphology and we have now carefully edited the discussion to be inclusive of possible interpretations.

3. The loss of Dia1 results in increased basal cell proliferation and hyperplasia (Fig 2C-D). How do the authors reconcile those findings to the ones shown in Fig. 4B, where the cells

populate the culture at a reduced rate and suggest that it is possibly due to reductions in cell proliferation?

Thank you for this question. In wild type conditions, the cell proliferation becomes suppressed in mature organotypic cultures. Compared to this, the initial proliferation rate to be suppressed in Dia1KD cells (Fig. S8) but, more importantly, their proliferation is not suppressed during the long term maturation of 3D organotypic cultures.

Thus, we believe the discrepancy in these data arises from the time scale of the cultures. The 2D experiments are short term, lasting no more than 4 days, and probe the proliferation in immature monolayers. The 3D cultures have been grown for 1-2 weeks, and probe the proliferation in mature organotypic models. We have now clarified this better in the text.

4. Roles of Dia1 in maintaining the basal cell identity. The authors conducted rescue experiments re-expressing the full-length Dia molecule in Dia1KD cells and conducted transcriptome analyses. However, the associated genes found in their transcriptomic analyses are related to keratinization, cornification, and genes related to differentiation, e.g., Notch. Fig. 3A, B. Where cells exposed to a calcium switch before the analyses?

We conducted the assay in low calcium, maintenance media, as has been performed by several other groups previously (Miroshnikova et al., 2018),(Totaro et al., 2017). Indeed, it is intriguing to us as well that all these early differentiation markers show signatures and suggest a different “priming” of the cells prior to Ca-induction.

5. Dia1 in differential cell sorting. Under normal conditions, are there any differences in Dia1expression levels associated with compartmentalized areas in the epidermis? How is Dia1 downregulated in the basal layer? Wouldn't the Dia1 negative cell be already committed to differentiation and later undergo the proposed delamination model once crowding by a neighboring cell division? Discuss these scenarios.

These are intriguing questions. The Sampath lab found that Dia1 is a target of mi198, which demonstrates an inverse staining pattern to that observed for Dia1 (suprabasal instead of basal) in stratified squamous epithelia (Sundaram et al., 2017) .However, the timing of miR198-dependent suppression of Dia1 is a mystery as is the possibility of post-translational degradation. For comparison, however, this is the case even for heavily studied differentiation markers like K10 let alone formins, with some reporting evidence of K10 being initially turned on within the basal layer and positive cells being quickly delaminated (Miroshnikova et al., 2018).

It is difficult to find histochemical staining of Dia1 of skin, esophagus or cornea in publications. A recent RNAseq study from the Kasper lab (Joost et al., 2018), however, indicates that Dia1 is downregulated 4-fold in Lgr6+ stem cells and their progeny in interfollicular epidermis 1 day after wounding of neighboring tissue. Interestingly, Lgr5+ stem cells and their progeny in the hair follicle upregulate Dia1 4-fold under the same conditions. As Lgr5 and Lgr6 progeny are both eventually found at later timepoints to contribute to re-epithelialization of the wound it suggests that Dia1 expression might be modulated in order to accomplish specific tasks. Hypothetically, horizontal migration of Lgr5+ progeny out of the hair follicle niche might be requisite to assume the sort of interfollicular transcriptional program required to repair

interfollicular tissue. Dia1 is known to support horizontal migration of keratinocytes. On the other hand, Lgr6+ progeny which are already interfollicular may have to alter their capacity for vertical movement or stratification. The Greco lab has beautifully demonstrated that, in mice, the leading edge advances over the wound bed as a multilayered structure with upward movement of cells being accelerated under those conditions. Our data would support the theory that reduction of Dia1 is one mechanism by which to achieve that sort of vertical movement. Why stratification seems accelerated under those conditions, nonetheless, remains unclear. Our tissue culture studies and 3D organotypic cultures could be argued to best represent tissue that is actively trying to re-epithelialize and, as such, may be particularly sensitive to modulation of Dia1 levels.

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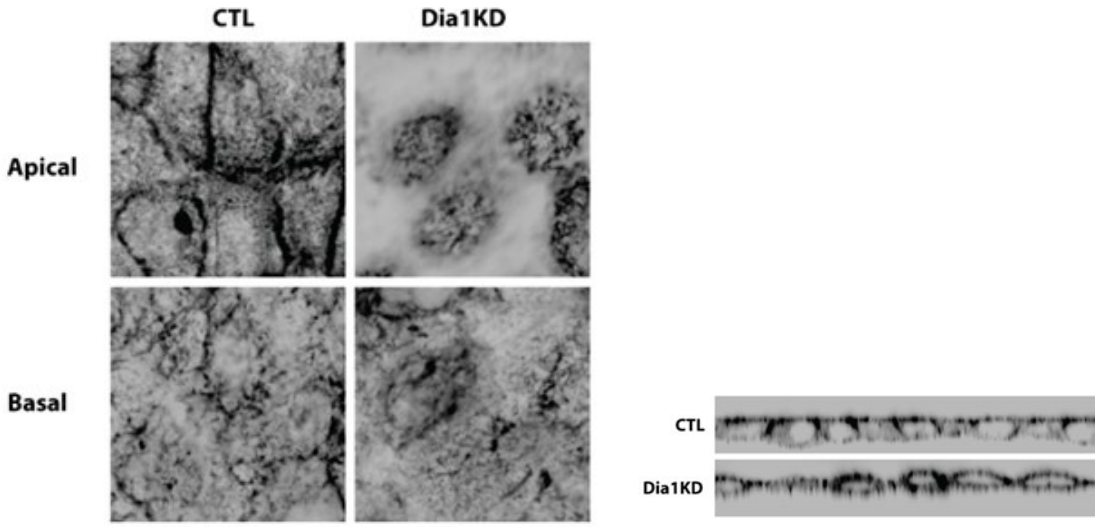
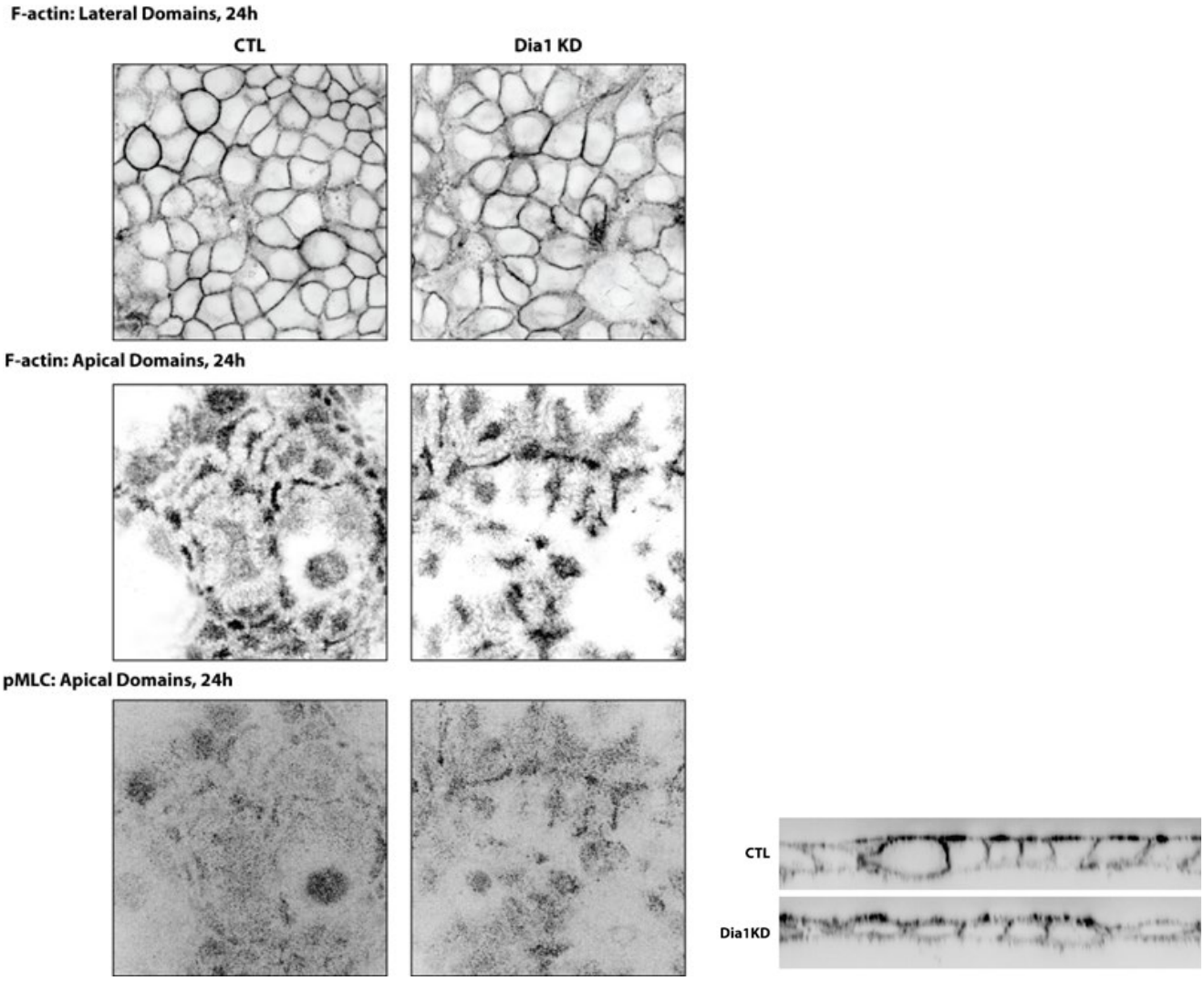
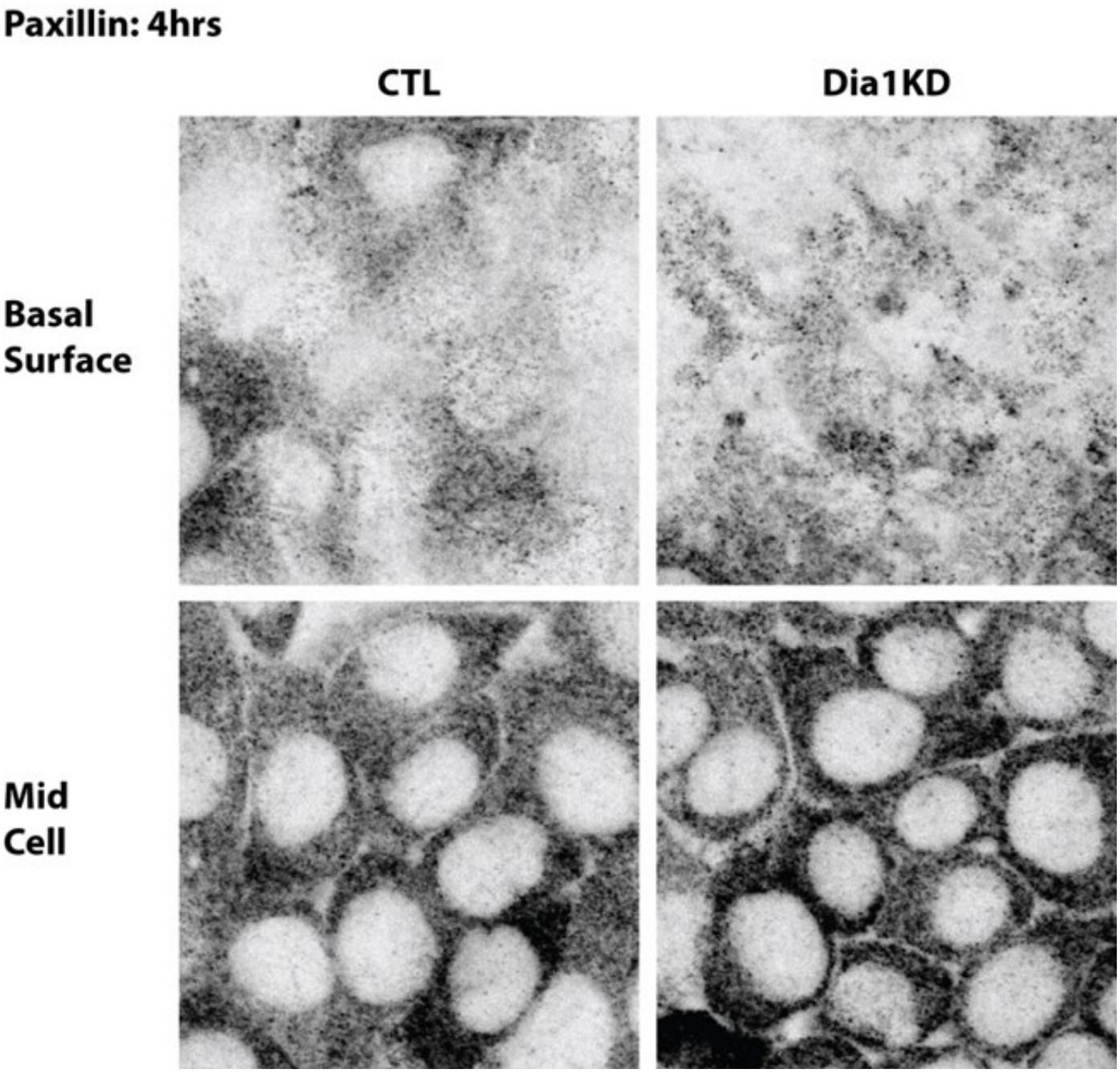
Reviewer Figures



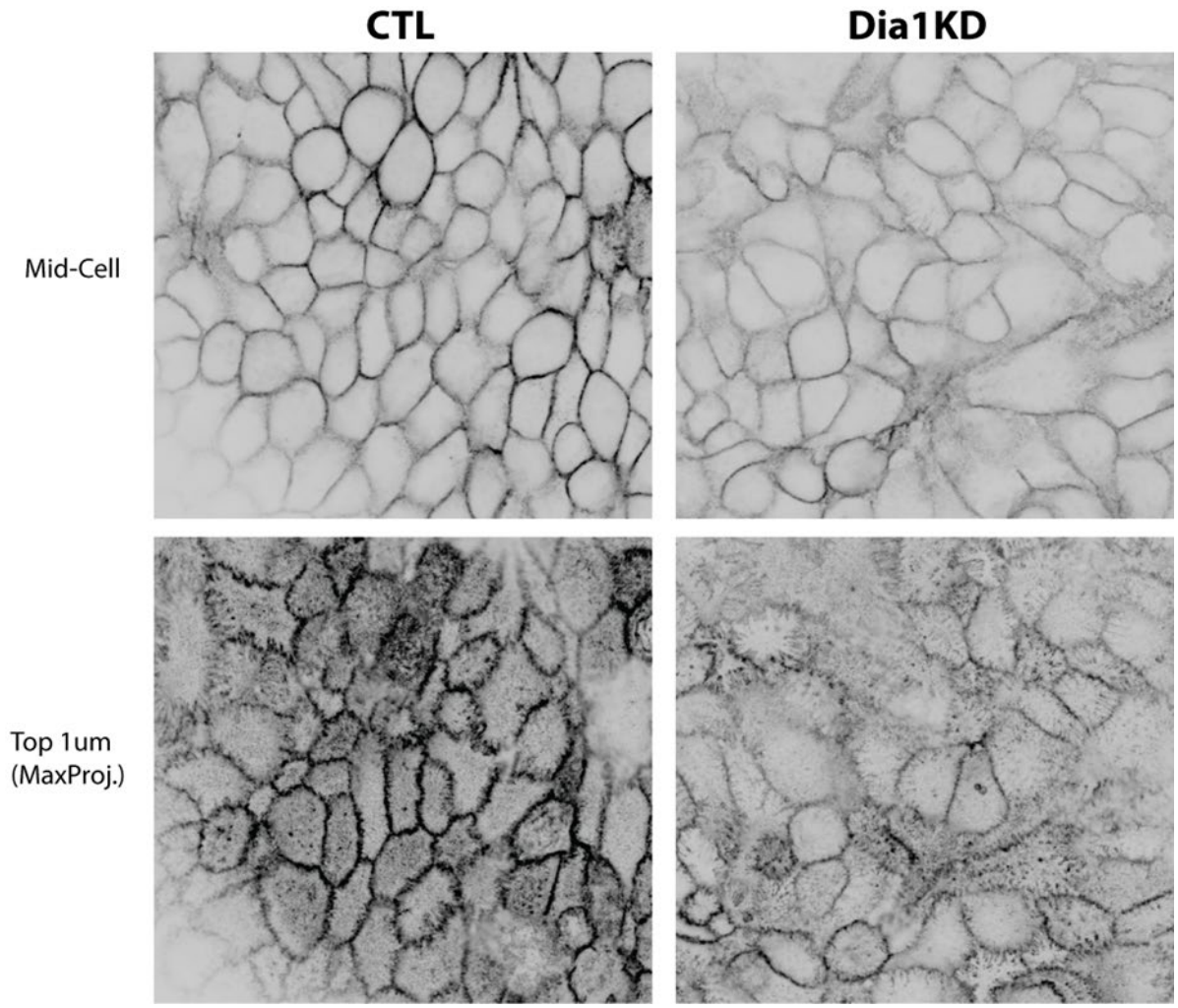
Reviewer Figure 1. Left panels. F-actin staining at the indicated planes of CTL and Dia1KD cells, 4hrs after plating on collagen gels. Right panels. Examples of orthogonal slices reconstructed from confocal z-slices of F-actin stains, illustrating the delayed vertical extension of Dia1KD lateral adhesive domains compared to CTL cells.



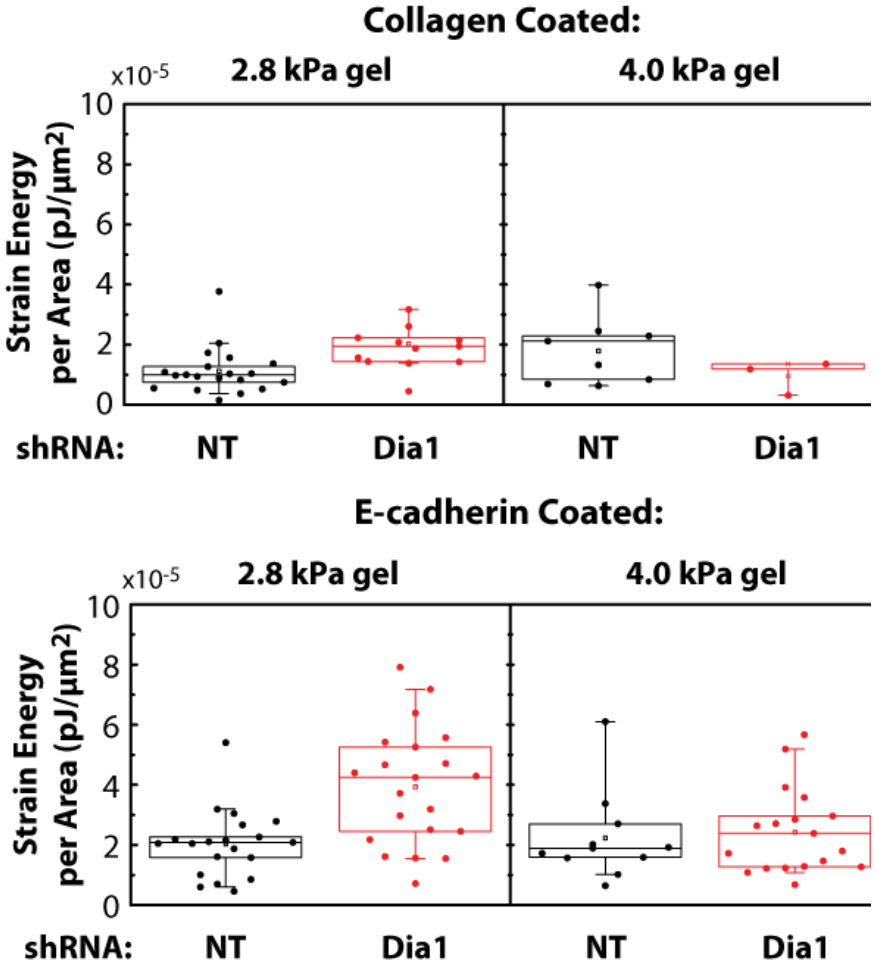
Reviewer Figure 2. F-actin and pMLC staining of CTL and Dia1KD cells at the indicated planes 24hrs after plating on collagen gels. Bottom panels are examples of orthogonal slices reconstructed from confocal z-slices of F-actin stained cells, illustrating that Dia1KD cells do eventually vertically extend their lateral adhesive domains.



Reviewer Figure 3. CTL and Dia1KD cells stained for paxillin and imaged at the basal surface or middle plane, 4hrs after plating on collagen gels.



Reviewer Figure 4. Staining of CTL and Dia1KD cells for E-cadherin at the middle plane of cells or near their apex, 24hrs after plating on collagen gels.



Reviewer Figure 5. Traction force microscopy measurements of single shCTL or shDIAPH1 cells plated on polyacrylamide gels of the indicated shear moduli. Top panels represent data acquired from collagen coated polyacrylamide. Bottom panels represent data derived from gels coated with E-cadherin ectodomains. Individual points represent individual cell measurements, box plots demonstrate 25-75 percentiles (boxes), median (line), mean (open square) and 5-95 percentiles whiskers.

December 13, 2021

Re: JCB manuscript #202101008R-A

Dr. Margaret Gardel
University of Chicago
University of Chicago 929 E 57th St GCIS E233
Chicago, IL 60605

Dear Dr. Gardel,

Thank you for submitting your revised manuscript entitled "Dia1 Coordinates Differentiation and Cell Sorting in a Stratified Epithelium". The manuscript has been seen again by the original reviewers whose full comments are appended below. While the reviewer continues to be overall positive about the work in terms of its suitability for JCB, an important issue remains to be addressed.

Specifically, reviewers #2 and 3 feel that you have not fully demonstrated the proposed differential expression of Dia1 under physiological/differentiation conditions. Reviewer #2 has suggested two possible approaches to addressing this issue (either immunostaining of epidermal tissue or, if that is not feasible, analysis of existing single-cell transcriptomic data). We hope that you will be able to address this remaining issue without too much trouble.

As you will also see, reviewer #3 has raised a couple of other issues. With regard to his/her second point (regarding the discrepancy between the 2D and 3D cultures), we feel that your explanation for this issue provided in your rebuttal and in the revised paper is sufficient. Finally, while we agree with the reviewer that a more complete understanding of the links between cell adhesion, proliferation/differentiation, and sorting (point #3) would undoubtedly enhance the impact of the study, we feel that such an extension is somewhat beyond the scope of the current paper and could be pursued in follow-up studies.

As you may know, 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 aim to submit the final revision within one to two months (though, if you are still experiencing COVID-related disruptions, please let us know and we can work something out), 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.

Sincerely,

Elaine Fuchs, PhD
Senior Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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

The authors have addressed most of the reviewer comments, and the manuscript has improved.

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

This paper has addressed most of my major concerns and has considerably improved with important additions also experimentally. I think the data are very interesting, with multiple interesting experiments to assess the role of Dia1 in differentiation and cell positioning, and the role of adhesion in this.
My only remaining point is the in vivo relevance: tthe data would gain significantly by showing by examining Dia1 staining in the epidermis. The authors refer to other papers, but this should, if good antibodies are available and they seem to be seeing Fig1A,

not be a big deal. Alternatively, there is a lot of single cell sequencing data available on the skin, that allow to differentiate between the differentiated and basal populations as well as the different hair follicle populations. These publicly available datasets could be explored for DIA1 expression.

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

Comments to the authors,
In this revised version, the authors have added additional data and reorganized their findings to streamline the ideas conveyed in their manuscript. I appreciate the efforts of the authors in modifying their manuscript to strengthen several of their conclusions. However, the revised version still does not address some of the caveats pointed out in the prior version, particularly in the following major points:
- Experimental data indicating that Dia expression levels are differentially expressed in compartmentalized areas of the epidermis or in their cultured control cells before and after delamination. These results will be helpful to support the potential relevance for Dia in maintaining basal cell identity and regulating cell sorting and differentiation.
- The discrepancies in cell proliferation exhibited by Dia KD cells in 2D cultures compared to the 3D ones are not addressed, despite the discussion of the potential causes in the text.
- The mechanistic connections between the cell adhesion angle, proliferation/differentiation, and sorting behavior need to be strengthened to substantiate their claims.

Dear Drs. Fuchs & Spencer,

We are extremely appreciative for the time you are spending to handle our manuscript #202101008R-A. Thank you. We are also grateful that you are allowing us to revise the manuscript a second time.

We now include in a new supplementary figure (Fig. S1) that shows immunohistochemical staining of human skin and tongue samples which are consistent with published data showing Dia1 concentrating in the basal layers of stratified epithelia. Included in Supplemental Figure 1 also images of Dia1 staining in a plucked, human hair follicle, showing its basal localization.

We agree that this data strengthens the manuscript and appreciate this suggestion from Reviewers #2 and #3. The changes to the text that describe this new data and the methods used to collect the data are indicated by yellow highlighting.

We hope you now find this work suitable for publication in the Journal of Cell Biology.

Best,

Margaret

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

The authors have addressed most of the reviewer comments, and the manuscript has improved.

We thank you for serving as a reviewer on our manuscript and that you feel we have addressed the reviewer concerns.

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

This paper has addressed most of my major concerns and has considerably improved with important additions also experimentally. I think the data are very interesting, with multiple interesting experiments to assess the role of Dia1 in differentiation and cell positioning, and the role of adhesion in this.
My only remaining point is the in vivo relevance: tthe data would gain significantly by showing by examining Dia1 staining in the epidermis. The authors refer to other papers, but this should, if good antibodies are available and they seem to be seeing Fig1A, not be a big deal.

Alternatively, there is a lot of single cell sequencing data available on the skin, that allow to differentiate between the differentiated and basal populations as well as the different hair follicle populations. These publicly available datasets could be explored for DIA1 expression.

Thank you for these suggestions. We now include in Supplemental Figure 1 immunohistochemical staining of human skin and tongue samples which are consistent with published images showing Dia1 concentrating in the basal layers of stratified epithelia.

Your suggestions also led us to consider hair follicles as a potentially interesting model for carrying out future studies on differential formin expression in complex epithelia. Included in Supplemental Figure 1 are new images of Dia1 staining in a plucked, human hair follicle. Note the localization of Dia1 to the columnar, basal cells of what we believe is the outer root sheath (ORS) in the region juxtaposed to the bulb. Expression appears to dramatically decrease in the second and third suprabasal layers, corresponding to those in which involucrin staining has been observed (Adly, M.A., Assaf, H.A. “Analysis of the expression pattern of involucrin in human scalp skin and hair follicles: hair cycle-associated alterations.” Histochem Cell Biol 138, 683–692 (2012). https://doi.org/10.1007/s00418-012-0986-4. Fig. 3c, f, g). Thus, this region of the hair follicle does appear to mimic what we have observed in organotypic cultures. For comparison, it is worth noting, in Supplemental Figure 1, that Dia1 expression extends into many layers of the bulb region which we suspect may correspond to hair matrix cells. This is consistent with the idea that altered formin distribution may contribute to tissue specification/compartmentalization.

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

Comments to the authors,
In this revised version, the authors have added additional data and reorganized their findings to streamline the ideas conveyed in their manuscript. I appreciate the efforts of the authors in modifying their manuscript to strengthen several of their conclusions. However, the revised version still does not address some of the caveats pointed out in the prior version, particularly in the following major points:
- Experimental data indicating that Dia expression levels are differentially expressed in compartmentalized areas of the epidermis or in their cultured control cells before and after delamination. These results will be helpful to support the potential relevance for Dia in maintaining basal cell identity and regulating cell sorting and differentiation.

We thank the reviewer for taking the time to carefully re-read our manuscript. We have now included a new data, in Fig. S1, to show varying Dia1 expression in human skin and tongue samples, which are consistent with published images showing Dia1 concentrating in the basal layers of stratified epithelia. Included in Supplemental Figure 1 are also images of Dia1 staining in a plucked, human hair follicle.

- The discrepancies in cell proliferation exhibited by Dia KD cells in 2D cultures compared to the 3D ones are not addressed, despite the discussion of the potential causes in the text.
- The mechanistic connections between the cell adhesion angle, proliferation/differentiation, and sorting behavior need to be strengthened to substantiate their claims.

We thank the reviewer for these additional points and note that the editor feels we addressed the first point in our previous revision and the second point is beyond the scope of this current work.

February 4, 2022

RE: JCB Manuscript #202101008RR

Dr. Margaret Gardel
University of Chicago
University of Chicago 929 E 57th St GCIS E233
Chicago, IL 60605

Dear Margaret:

Thank you for submitting your revised manuscript entitled "Dia1 Coordinates Differentiation and Cell Sorting in a Stratified Epithelium". We have now had an opportunity to assess your revised manuscript and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes the abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, materials and methods, figure legends, references, tables, or supplemental legends. You are currently below this limit but please bear it in mind when revising.

2) 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, including cropped gels. Thus, you will need to provide markers for the gels in figure S13A. Please also provide scale bars for the images in figures S1A-E, S4A, S4B, S12A, S13B, and S14A.

3) 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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) 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.

6) 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.).

7) 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.

8) Supplemental materials: There are usually fairly strict limits on the allowable amount of supplemental data. Articles may usually have up to 5 supplemental figures. Needless to say, you exceed this limit but, given the circumstances, we will be able to give you the extra space this time. However, please do not add to the current total.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material (in addition to the supplementary figure legends) should appear at the end of the Materials and methods section. Please see any recent JCB paper for an example of this.

9) 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."

10) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

11) 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.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Elaine Fuchs, PhD
Senior Editor
The Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology
