



Ca²⁺ transients in melanocyte dendrites and dendritic spine-like structures evoked by cell-to-cell signaling

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March 14, 2019

Re: JCB manuscript #201902014

Dr. Sanford M Simon
Rockefeller University
Cellular Biophysics
1230 York Avenue
New York, New York 10065

Dear Dr. Simon,

Thank you for submitting your manuscript entitled "Ca²⁺ transients in melanocyte dendrites and dendritic spine-like structures evoked by cell-to-cell signaling". Thank you for your patience with the review process, and we are sorry we could not communicate our decision to you earlier. Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that both expert referees provided detailed and constructive comments indicating interest in the studies of calcium transients upon melanocyte-keratinocyte contacts but raised numerous and significant concerns about the data and manuscript presentation. Some of the issues raised by both referees include lack of controls, unclear stainings or requests for additional stainings, better characterizations of the cell layers, questions about the protocols for the calcium experiments. Rev#1 had questions about the time dynamics of the keratinocyte-melanocyte interactions (Fig 2) and calcium transients (Fig 3). Echoing some of Rev#1's questions about the possible enrichment of calcium transients in dendrites, Rev#2 did not feel that comparable data are being compared to establish this claim (#1). For this ref, the data implicating ET-1 in calcium transients was not clear and strong enough (#2-3, also #5). Importantly, Rev#2 asked if the calcium transients seen in melanocyte dendrites depend on the contacts with keratinocytes or keratinocyte-secreted factors (#4) and requested mechanistic insight into the contribution of contacts between melanocyte dendrites and keratinocytes to Ca²⁺ transients in melanocytes and into the discrepancy between transients observed more in dendrites than in the cell body. Rev#2 asked if filopodia have calcium transients and what the importance of dendritic spines to these observations is (#7). Rev#2 additionally discussed the lack of evidence that the calcium transients have any particular function in melanocytes locally or globally (#6), indicating that the comparison to neuronal systems was therefore unfounded.

We discussed this feedback in depth. We feel that these points, from experts in the field, are valid and important. In our view, the technical and experimental points from the reviewers would need to be addressed rigorously and significantly for the data to offer sufficient support for the core conclusions to warrant publication in JCB. While we would not require functional analyses of these currents for resubmission to the journal, we strongly feel that addressing all other points would be needed to bolster the observations and refine the mechanistic analyses, as guided by the reviewers' comments. Further characterizations of the spatiotemporal dynamics of the calcium transients and melanocyte-keratinocyte interactions and addressing the reviewers' concerns about the lack of clarity as to what is shown and what was done experimentally would be needed. We

realize that a significant amount of work would be needed to tackle all the reviewers' points and therefore strongly encourage you to send to us a revision proposal if you are interested in revising and resubmitting to JCB. Should you submit a revision plan, we would discuss this rebuttal editorially and possibly get referee input to ensure that you do not embark on time- and resource-consuming revisions that may not be sufficient for a successful resubmission. For instance, we can envision that discussing how to address the mechanistic questions from Rev#2 could be beneficial to ensure that we are all on the same page as to the degree to which the work and analyses need to be bolstered for further consideration at JCB. We would aim to consult the same referees at resubmission; to move forward for further consideration at the journal, we would look for strong enthusiasm and support from the referees and would seek feedback as to whether the core conclusions are stronger, with better mechanistic evidence as described by Rev#2. We would also expect that the claims related to neuronal systems functionally be removed in the absence of functional studies of calcium transients in melanocytes.

We hope that our expectations for further consideration at JCB are clear. Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

Further, if you would prefer to submit this manuscript elsewhere at this stage, we are also happy to transfer your reviewer comments to any other journal upon request. Many journals will accept transfer of reviewer comments from JCB, such as Molecular Biology of the Cell and Journal of Cell Science. If you decide to submit this work to a journal that will accept transfer of reviewer comments, simply email the JCB editorial office and we will initiate the transfer on your behalf. You also have the option to internally transfer your manuscript to any RUP journal: JEM, JGP, or our open access journal Life Science Alliance (<http://www.life-science-alliance.org/>), launched as a collaboration between RUP, EMBO Press and Cold Spring Harbor Lab Press. Please let us know if you would like to transfer to LSA and if you'd like to discuss the level of revision, if any, needed for publication in LSA. Please also feel free to reach out to LSA Executive Editor Andrea Leibfried directly to discuss a potential transfer.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <http://jcb.rupress.org/site/misc/fora.xhtml>. All figures in accepted manuscripts will be screened prior to publication.

*****IMPORTANT:** It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Cédric Blanpain, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

Melanocytes and keratinocytes are the major cellular components of the epidermis and it is a well-established fact that their interactions allow for proper skin pigmentation and protection against UV radiation. However, how these two cell types interact with each other, and what types of cell signaling events are influenced as a result of the close proximity between these two cells in the epidermis still remains unclear. In this study, Belote and Simon attempt to further characterize the melanocyte-keratinocyte interaction using an in vitro cell co-culture system containing melanocytes and keratinocytes isolated from human neonatal foreskin. More specifically, they focus on the interaction between keratinocytes and the melanocyte dendrites. They show that keratinocytes contain membrane processes that are in close proximity to/contact melanocyte dendrites and that the dendrites contain spine-like projections that associate with the keratinocytes. Co-culturing of melanocytes and keratinocytes results in an increase in Ca^{2+} transients in melanocytes which is suggested to be attributed to keratinocyte-produced endothelin-1 and acetylcholine. These spine structures are also the sites of Ca^{2+} transient initiation. The results of the structural and morphological studies in cell culture are also examined in vivo in intact neonatal foreskin.

Major Comments:

While this work is interesting, there are a significant number of points that need to be clarified and addressed.

Most notably, the results of the study are not sufficiently explained in detail in the text, but are rather glossed over quite quickly. The unclear textual description along with the description in the

figure legends is not enough to adequately guide the reader. This contributes to an overall lack of clarity of the results, which is a significant problem.

The methodologies are also not clear at times. Most notably, it is not clear in which system and under which conditions, CaCl_2 is present.

Many of the panels are too small and very difficult to understand, especially the EM images and the kymographs, from which no information can be drawn.

Specific Comments:

Figure 1

- Why is there no keratinocyte marker in Fig. 1A? This is an important control that should be done.
- The "two color" staining is not clear. The cKit antibody (Thermo, CD11705) is an APC-conjugated antibody. Why is there no red colour to the staining? Did the authors use an unconjugated antibody?
- A higher magnification of this image is needed to clearly see the Tyrp1 and cKit staining.
- The authors state that there are multiple cell layers with stratification and different characteristics within the cell culture. What is the staining pattern for the K10 and K14 in the other layers within the culture?
- Where are the melanocytes in this co-culture? Panel C suggests that they contact the keratinocytes, but in which layer? The melanocyte distribution within the different layers needs to be demonstrated.
- With regards to 1E, from which layer of the co-culture does this pattern of staining come? What is the E-cadherin staining in the other layers? Is there colocalization of E-cadherin with catenin proteins? This would strengthen the argument that there is functional cell-cell adhesion. What is the status of P-cadherin?
- A clear staining to differentiate the melanocytes and keratinocytes in 1E should be done. What does the phalloidin look like for each cell type in this co-culture at different CaCl_2 concentrations?
- While 1F shows that the bottom layer of the co-culture contains K14 positive cells and essentially no K10 positive cells, the top layer of cells shows only a few sparse K10 positive cells. Is this truly representative of a layer of cells? Are there other cells in this top layer?
- Why did the authors not use a confocal microscope for imaging the different layers in the cell culture?
- Are the authors referring to panel 1C when stating that "melanocytes exhibited morphologies similar to those from intact epidermal sheets" rather than 1F as stated in the text?

Figure 2

- While the authors state that "keratinocytes had processes that extended from the cell surface and contacted and wrapped adjacent melanocyte dendrites," the images provided do not clearly show a wrapping by these projections. A 3D image would be necessary to demonstrate wrapping of the dendrite.
- A lower magnification of 2A would be helpful to have an idea of how the keratinocyte and melanocyte in question look. A higher magnification image would be useful to better see this sort of cell-cell contact.
- Are the keratinocytes that are forming these membrane envelopes K10 or K14 positive? Where is E-cadherin in relation to these keratinocyte processes?
- The authors claim that the interaction between the keratinocyte processes and the melanocyte dendrites were stable, but they only looked for a period of 90 minutes. Was this interaction still occurring over a longer period of time?

- It is not clear what the authors are looking at in panel C. The distance, d , is defined as the distance between the "farthest edge of the melanocyte dendrite (short yellow line) to the closest edge of the keratinocyte cell body." What do they mean by the "farthest edge" of the dendrite? A lower magnification showing the entire dendrite would be useful here. The small yellow line does not appear, to the reviewer, to be at the farthest edge of the dendrite.
- Furthermore, the graph in panel C shows that the distance, d , decreases over the 90 minutes. Does this mean that the interaction is stronger or just that the dendrite is closer to the keratinocyte cell body? What is the farthest distance where the authors observed an interaction?
- The images in panels D and E are not at all clear. The images are hard to see (no proper contrast) and too small, making them incomprehensible. The authors should, at the very least, make clear outlines of where the melanocytes and keratinocytes are. It is also unclear what the * is actually referring to.
- The authors also observed pools of small vesicles in the cytoplasm of keratinocytes that were adjacent to the melanocyte dendrites, which "suggested that melanocyte dendrites might receive localized signaling from individual keratinocytes." Are there no vesicles in the cytosol of keratinocytes that are not juxtaposed to the melanocyte dendrite?

Figure 3

- Were the experiments measuring the calcium transients done in co-cultures in the presence of CaCl_2 ?
- In 3A, the co-cultures are presented based on the different donors. Is this an important consideration? Why were there 6 co-cultures from a particular donor and only 1 from another? Did the authors try mixing keratinocytes and melanocytes from different donors?
- Why did the authors choose to examine the transients in only a 2.5-minute window? What happens if they look longer?
- Why was the data for whole cell and local transients pooled? This is confusing and unclear.
- The authors claim that "The number of local dendritic transients detected during 2.5 minutes ranged from 0-72 per melanocyte (Fig. 3E)." However, based on the data from the graph, the vast majority of cells have between 0 and 10 transients, with anything more being quite uncommon.
- Panel E takes data from 27 co-cultures from 7 donors whereas panel A takes data from 22 co-cultures and 6 donors. The phototypes of the donors can be of importance this should be mentioned. Why the discrepancy? Are the 22 donors in 3A included in the 27 from 3E?
- Panel 3E also shows that approximately 600 out of 1793 melanocytes had 0 transients in the 2.5 min period. Is this consistent with the data from 3A?
- Why were only 3 co-cultures from 3 skin donors analyzed for the analyses in 3F?

Figure 4

- In panel 4B, at 3.25s, the signal appears to be less than at 2.75 and 3.75s. Why is this the case and why is the graph for 3.25s not presented in panel C?
- In panel 4C on the graph to the right, what are the authors trying to show?
- Panel 4B presents data from 2.25 to 7.5 seconds while panel D presents from 10.25 to 24.95 seconds. Is there any significance to these different times or durations?
- The data from panel 4F is incomprehensible. What are the authors trying to show?
- In the text, the authors claim that "In those dendrites that had multiple Ca^{2+} transients over time, the repeat transients initiated from the same region of the dendrite (Fig. 3F)." Do the authors mean 4F and not 3F?

Figure 5

- What are unlabeled melanocytes?
- Panel 5B gives the scale on the y-axis as % pre-treatment level. What does this mean? What is

the pre-treatment? Treatment of CaCl_2 should increase the number of cells with transients, so this percentage should be higher than 100.

- As there are a great deal of results and they are not all clearly explained, what is the result when melanocytes are cultured alone in the presence of CaCl_2 ? How many calcium transients are there and how does this compare to the co-culture system?
- Why are the scales different between panels C and D?
- What is the Control sample in panel 5D and where is the CaCl_2 1.06mM?
- Why is there such a considerable difference between the distribution of the blue bars from panels 5C and 5D? Again, the authors must be clear here. What is the pre-treatment and what is a treatment of 0 mM CaCl_2 ?
- The title of this figure is "External and internal Ca^{2+} pools contribute to melanocyte dendritic Ca^{2+} transients." Were the measurements taken exclusively from the dendritic transients?

Figure 6

- The authors state that "the localized Ca^{2+} transients in melanocytes elicited by ET-1 (Figure 6A) resembled the spontaneous Ca^{2+} transients in co-cultures of melanocytes and keratinocytes without the addition of exogenous ET-1 (Fig. 4)." Could the authors please clarify where in Fig. 4 a graph like that in panel 6A is shown? Are they referring to Fig. 3? If so, where is the similarity?
- Were the experiments with ET-1 done in the presence of CaCl_2 ?

Figure 7

- For both panels A and C: what is the pre-treatment? What is the control?
- The authors have not demonstrated that their antagonists work efficiently.
- In panel 7B, what is the level of knockdown for each siRNA?
- Knocking down of the endothelin receptors in the melanocytes does not show clearly that the keratinocytes are responsible for the ET-1 signal. In order to clearly demonstrate this, expression of the EDN1 gene must be silenced in keratinocytes and the absence of the protein must be confirmed. In this way, the role of keratinocyte ET-1 will be accurately assessed.
- A similar principle is true for the acetylcholine effect. Knockdown of (for example) choline acetyltransferase in keratinocytes would inhibit the production of the acetylcholine and therefore clearly indicate whether or not keratinocyte acetylcholine is important for the melanocyte Ca^{2+} transients.

Figure 8

- What is the significance of the graph in panel 8C?
- The authors state that "some dendrites having no detectable spine-like structures," but this is not reflected in panel 8D.
- Panel 8F does not show a clear interaction being made between the melanocytes and the keratinocytes. A confocal image showing overlap or a 3D reconstruction would be necessary.
- Do the dendrites interact with the keratinocyte envelope structures when there is re-shaping of the spines (panel 8H)?
- Are the spine-like structures present on the dendrites of melanocytes when they are alone in culture?
- Panel 8F also shows that the keratinocyte envelope structures clearly do not wrap around the melanocyte dendrite. Perhaps a different word should be used to describe this phenomenon.
- Do the keratinocyte membrane structures interact with the melanocyte spine-like structures?

Figure 9

- The kymographs in panel C are very hard to see and discern.
- How many of the total dendritic transients were found in clear, spine-like structures?

- Are these small, unresolvable spine-like structures in close proximity/contact with keratinocytes?

Figure 10

- The authors used the lack of keratin filaments as a marker for melanocytes, but this is not accurate, as melanocytes may express some keratins.
- Where are the spine-like structures in panel 10B?
- Figure 10C needs to show the shape/size of the spine-like structures from melanocytes in culture in comparison to those found in the skin side-by-side for any meaningful comparison.

Minor Comments:

As mentioned above in specific sections, the results are mis-referenced in the text at times, which leads to confusion.

There are some typographical errors that should be fixed.

Page numbers should be added.

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

Belote et al studied change of Ca^{2+} transients in melanocyte dendrites driven by neighboring keratinocytes in a co-culture system. They showed that melanocytes dendrites and keratinocyte protrusions closely interact. Co-culture with keratinocyte promotes Ca^{2+} transients in melanocyte dendrites, which is enhanced by ET1 and ACTH, known to be produced by keratinocytes. While the study is carefully performed with large amount of data at a high resolution, providing good basis for a culture system to study melanocyte/keratinocyte signal transduction, there were several concerns such as inconsistent data, lack of data showing the keratinocyte/melanocyte interaction rely on dendrites/compartments, functional data and mechanistic view and similarity to neuron are weak.

Major concerns:

1) It seems unfair to compare Ca^{2+} transients in certain small areas of dendrites (Fig. 3B and Fig. 4) to whole cell transients (Fig. 3C) and conclude that spontaneous Ca^{2+} transients are compartmentalized mainly in dendrites. It makes more sense to compare Ca^{2+} transients in any areas of dendrites to those in similar size of areas in cell body to show enrichment of transients in dendrites but not cell body. There seems to be no data to show there is more local transients in dendrites than local transients in cell body.

2) SFig.1 showed that in both melanocyte/keratinocyte co-culture and melanocyte single culture, there is no difference in the Ca^{2+} transients in between keratinocyte culture medium (do not contain ET-1) and melanocyte culture medium (contain final concentration of 10nM ET1). However, addition of 10nM ET1 in co-culture (Fig. 6) in keratinocyte culture medium and melanocyte single culture (SFig.2) can induce more robust Ca^{2+} transients. These data seem inconsistent about the role of ET-1 in inducing more Ca^{2+} transients.

3) Fig. 6A showed that with addition of ET-1, there is also quite obvious increased Ca^{2+} transients in cell body. Does ET-1 induce more cell body Ca^{2+} transients than baseline and ACTH?

4) It wasn't clear whether melanocyte Ca^{2+} transients rely on the contact of their dendrites with keratinocytes, or keratinocyte secreted factors, or both. If both, which is more important/required? It was already known that keratinocyte secreted factors can induce change in melanocyte Ca^{2+} transients, so it is more important to show stronger data that the direct dendrite contact is important. SFig.1 seems to show some data for this (culture of melanocytes with keratinocytes conditioned medium), but it is difficult to interpret because there is very few description of this experiment.

5) There is no mechanism about why the contact between melanocyte dendrite and keratinocyte is important for Ca^{2+} transients in melanocytes. Also no mechanism why more transients were observed in dendrites than cell body. Is there evidence that more ET1 and ACTH receptors are located in the dendrites?

6) The whole manuscript showed "compartmentalized Ca^{2+} transients" but did not show whether this elicits local changes but not global changes in melanocytes. Most known function of ET1 and ACTH in melanocytes are through changing transcription of several melanocytic genes, which needs to go to nucleus and cannot be a local change, even if the signals were received locally. Without such data, it is difficult to conclude that melanocyte dendrites mimic neuron in compartmentalized response to signals.

The authors mentioned the similarity of melanocytes to neurons in their dendrites properties and functions many times in the text, but there is no neuron data as control.

7) Spine structures on dendrites were studied in 1/3 of the paper, but it is not clear what is the importance/difference of this structure compared to dendrites and filopodia on dendrites in Ca^{2+} transients or other functions. Do filopodia also have Ca^{2+} transients? How about interaction of filopodia with keratinocytes?

Minor concerns:

In Fig. 1C, it seems that many keratinocytes were not labeled by GFP. What percentages of melanocytes and keratinocytes were labeled by fluorescent proteins in this culture system?

What is the cause of baseline Ca^{2+} transients studied in Fig. 3 and 4? Is it caused by certain supplements in the keratinocyte culture medium, such as BPE, Igf1 and Egf?

How many layers of keratinocytes were in the culture system and is it comparable to human? In Fig. 1F, 3D image or Z-stack images showing each layer should be added to help the interpretation of data. What is the distribution of melanocytes in multiple layers of keratinocytes in the culture system? Are they mainly located in the basal layer as in human?

Fig. 1A, how can TRP1 and cKit both in green?

In Fig. 2D and E, it is difficult to appreciate how keratinocyte processes envelope dendrites of melanocytes. 3D reconstruction of TEM images acquired on serial sections (or something like SFig. 4) may be needed. It is also not clear based on what evidence the keratinocyte processes and melanocyte dendrites were identified on the TEM images, which may be helped by showing lower magnification images of the same area showing more defining details of both cell types.

Figure number should follow the order they appear in the text. For example, Fig 3 and 4 were quite mixed in their order in text.

1st Revision - Authors' Response to Reviewers: September 19, 2019

Dear Cédric Blanpain, Melina Casadio, and Jodi Nunnari,

Thank you for providing clarity to our remaining questions. We have gone back and modified our response to the referees based on your feedback. In addition, we have revised the manuscript so that all comparisons of melanocytes to neurons and glia are in the discussion and not in the abstract, introduction and results.

We hope that you find our revisions acceptable to The Journal of Cell Biology. Thank you again for your input and time.

Best,
Sandy Simon

To the referees,

We would like to thank the referees for their comments. We have taken them all seriously and we believe that this is a clearer and stronger manuscript as result.

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

Melanocytes and keratinocytes are the major cellular components of the epidermis and it is a well-established fact that their interactions allow for proper skin pigmentation and protection against UV radiation. However, how these two cell types interact with each other, and what types of cell signaling events are influenced as a result of the close proximity between these two cells in the epidermis still remains unclear. In this study, Belote and Simon attempt to further characterize the melanocyte-keratinocyte interaction using an in vitro cell co-culture system containing melanocytes and keratinocytes isolated from human neonatal foreskin. More specifically, they focus on the interaction between keratinocytes and the melanocyte dendrites. They show that keratinocytes contain membrane processes that are in close proximity to/contact melanocyte dendrites and that the dendrites contain spine-like projections that associate with the keratinocytes. Co-culturing of melanocytes and keratinocytes results in an increase in Ca^{2+} transients in melanocytes which is suggested to be attributed to keratinocyte-produced endothelin-1 and acetylcholine. These spine structures are also the sites of Ca^{2+} transient initiation. The results of the structural and morphological studies in cell culture are also examined in vivo in intact neonatal foreskin.

Major Comments:

While this work is interesting, there are a significant number of points that need to be clarified and addressed.

Most notably, the results of the study are not sufficiently explained in detail in the text, but are rather glossed over quite quickly. The unclear textual description along with the description in the figure legends is not enough to adequately guide the reader. This contributes to an overall lack of clarity of the results, which is a significant problem.

The methodologies are also not clear at times. Most notably, it is not clear in which system and under which conditions, CaCl_2 is present.

Many of the panels are too small and very difficult to understand, especially the EM images and the kymographs, from which no information can be drawn.

Response: We thank the reviewer for highlighting where we need to be clearer and present more information for how we have conducted each experiment. We have revised the text and figures to be more specific about our experimental conditions. We have addressed each specific figure comment on this matter below. We have also elaborated and clarified the language regarding our results for areas of confusions that have been brought to our attention by both reviewers. Please see the detailed responses for each item below.

Figure 1:

- Why is there no keratinocyte marker in Fig. 1A? This is an important control that should be done.

Response: We have added a new supplemental figure (Supplemental Figure 1) and included a representative image of an epidermal sheet stained for the basal keratinocyte marker alpha 6 integrin and another melanocyte marker tyrosinase (TYR). We did not originally include the keratinocyte marker because separation of the epidermis from the dermis by dispase is a standard procedure for isolating epidermal sheets used routinely in our lab and in other labs for isolating both melanocytes and basal keratinocytes. We also routinely use this method to isolate intact epidermal sheets for immunofluorescence and microinjection. Every time we have probed for keratinocytes and/or melanocytes, we have observed an intact basal layer of cells in the epidermal sheet. When imaging only melanocytes, a visual inspection of the cell layers in infrared DIC (and/or with Hoechst) was used to verify that both keratinocytes and melanocytes were present.

- The "two color" staining is not clear. The cKit antibody (Thermo, CD11705) is an APC-conjugated antibody. Why is there no red color to the staining? Did the authors use an unconjugated antibody?

Response: We wanted to provide a comprehensive labeling of the melanocytes. Thus, we labeled melanocytes both with mouse anti-TRP1, for melanosomes, and mouse anti-cKit, for plasma membranes. One secondary antibody (anti-mouse IgG) was used to detect both primary antibodies. Reviewer 1 is correct that the cKit antibody is directly conjugated to APC (there is also an unlabeled version of the same antibody available from the supplier). However, since we were using a secondary antibody (anti-mouse H+L chain IgG) that recognized both TRP1 and cKit we did not utilize the APC on the cKit. We have revised the methods to be clearer (see lines 371-374). We have also described the melanocyte-specific antibody cocktail (the combination of TRP1 and cKit) in the new Supplemental Figure 1.

- A higher magnification of this image is needed to clearly see the Tyrp1 and cKit staining.

Response: We have included this in the new Supplemental Figure 1A.

- The authors state that there are multiple cell layers with stratification and different characteristics within the cell culture. What is the staining pattern for the K10 and K14 in the other layers within the culture?

Response: We thank the reviewer for pointing out that we need to be clearer with our choice of words. The multiple cell layers refer to 2 to 3 layers. The bottom layer has K14 positive keratinocytes with any keratinocyte above that layer being K10 positive. We have revised the text to be clearer about this. Please see lines 57-60, 744-745.

- Where are the melanocytes in this co-culture? Panel C suggests that they contact the keratinocytes, but in which layer? The melanocyte distribution within the different layers needs to be demonstrated.

Response: Melanocytes are on the bottom layer of the co-culture. Their dendrites interact with keratinocytes in the bottom layer as well as keratinocyte above the bottom layer. We have revised the text (lines 57-58, 64-67) to include this information as well as modified Figure 1 to include a new panel, 1G, to show their localization within the co-culture.

- With regards to 1E, from which layer of the co-culture does this pattern of staining come?

Response: This image comes from the bottom layer. We have revised the figure legend to include this information. Please see lines 746-747.

- What is the E-cadherin staining in the other layers?

Response: Ecadherin is in all layers of the co-culture. Please see revised Figure 1, panel 1D.

- Is there colocalization of E-cadherin with catenin proteins? This would strengthen the argument that there is functional cell-cell adhesion.

Response: Yes there is co-localization of E-cadherin and beta-catenin. Please see revised figure 1, panel 1D

- What is the status of P-cadherin?

Response: We have revised figure 1 (panels 1E, 1F) to include P-cadherin staining which shows that co-cultures express p-cadherin and it is localized at sights of cell-cell contact.

- A clear staining to differentiate the melanocytes and keratinocytes in 1E should be done. What does the phalloidin look like for each cell type in this co-culture at different CaCl₂ concentrations?

Response: We have revised figure 1 (panels 1E, 1F) to include melanocyte staining for ECAD, PCAD and phalloidin staining in both high and low CaCl₂ conditions.

- While 1F shows that the bottom layer of the co-culture contains K14 positive cells and essentially no K10 positive cells, the top layer of cells shows only a few sparse K10 positive cells. Is this truly representative of a layer of cells? Are there other cells in this top layer?

Response: Yes, this is representative of what we observe. The bottom layers contain K14 positive keratinocytes and the K10 positive cells are only in the upper layers. The number of cells and intensity of the K10 staining in the top layer(s) vary within the culture and from culture to culture. We have revised the text (lines 57-60) and figure legend to address this.

- Why did the authors not use a confocal microscope for imaging the different layers in the cell culture?

Response: Imaging the different layers can be achieved by confocal or epifluorescence microscopy. There are a few reasons why we went with deconvolution rather than confocal microscopy. First, deconvolved epifluorescence images have comparable spatial resolution to those taken on a confocal microscope and can be used for 3D analysis. Second, confocal scanning through the layers requires much higher laser intensity which meant much greater rates of photobleaching, which might result in missing the fine processes.

For our purposes, we did not want to alter the 3D architecture of the culture by mounting with a traditional mounting media that contain anti-fade reagents. We have found that even an uncured viscous mounting media (like Prolong Gold from Molecular Probes) alters the three-dimensional organization of the cells by significantly flattening cells. Since the co-cultures were imaged in a PBS solution, which made them more susceptible to photobleaching, we did not want the additional photobleaching that occurs with the more intense excitation required of confocal.

- Are the authors referring to panel 1C when stating that "melanocytes exhibited morphologies similar to those from intact epidermal sheets" rather than 1F as stated in the text?

Response: We thank the reviewer for catching this error. Yes, we will revise the text according to the new Figure 1 and Supplemental Figure 1 panel arrangements. Please see lines 64-65 and new corresponding figures.

Figure 2:

- While the authors state that "keratinocytes had processes that extended from the cell surface and contacted and wrapped adjacent melanocyte dendrites," the images provided do not clearly show a wrapping by these projections. A 3D image would be necessary to demonstrate wrapping of the dendrite.

Response: We have revised figure 2 by adding panels 2F-H showing a 3D reconstruction of keratinocyte processes in intact neonatal foreskin.

- A lower magnification of 2A would be helpful to have an idea of how the keratinocyte and melanocyte in question look. A higher magnification image would be useful to better see this sort of cell-cell contact.

Response: We have revised the figure to include both a low magnification image and a higher magnification image in panel 2A. We have also included corresponding Z,XY images to better depict the contact between the two cells.

- Are the keratinocytes that are forming these membrane envelopes K10 or K14 positive? Where is E-cadherin in relation to these keratinocyte processes?

Response: When staining co-cultures for E-cadherin, there is so much signal throughout the culture on both melanocytes and keratinocytes that it was impossible to localize it relative to the keratinocytes. In the intact skin both basal and suprabasal keratinocytes have processes that wrap around melanocyte dendrites. We have observed processes on keratinocytes in the bottom layer and second layer of the culture. The bottom layer is composed of K14 positive keratinocytes and the top layers are K10 positive keratinocytes.

- The authors claim that the interaction between the keratinocyte processes and the melanocyte dendrites were stable, but they only looked for a period of 90 minutes. Was this interaction still occurring over a longer period of time?

Response: We did not image past 90 minutes and thus cannot make any claims regarding interactions over longer periods of time. We have revised the text to say that they are stable on the time course of an hour. Please see lines 74-75.

- It is not clear what the authors are looking at in panel C. The distance, d , is defined as the distance between the "farthest edge of the melanocyte dendrite (short yellow line) to the closest edge of the keratinocyte cell body." What do they mean by the "farthest edge" of the dendrite? A lower magnification showing the entire dendrite would be useful here. The small yellow line does not appear, to the reviewer, to be at the farthest edge of the dendrite.

Response: We have revised the figure legend for figure 2C to be clearer about what distance " d " is. We thank the reviewer for pointing out a mistake in the figure legend.

- Furthermore, the graph in panel C shows that the distance, d , decreases over the 90 minutes. Does this mean that the interaction is stronger or just that the dendrite is closer to the keratinocyte cell body? What is the farthest distance where the authors observed an interaction?

Response: The decrease in distance over 90 minutes shows that the dendrite has moved closer to the keratinocyte cell body. We do not think a claim can be made about the strength of the interaction. This data shows that the melanocyte dendrite and keratinocyte process remained continuously in contact even while the dendrite underwent spatial relocation and underwent morphological changes.

- The images in panels D and E are not at all clear. The images are hard to see (no proper contrast) and too small, making them incomprehensible. The authors should, at the very least, make clear outlines of where the melanocytes and keratinocytes are. It is also unclear what the * is actually referring to.

Response: We thank the reviewer for their suggestion and have made the EM images larger with better contrast and labeling to depict where each cell type is in the image. Please see figure 2D,E. Keratinocytes (k) are pseudo colored in different shades of green/blue and melanocyte dendrites are pseudo colored purple.

- The authors also observed pools of small vesicles in the cytoplasm of keratinocytes that were adjacent to the melanocyte dendrites, which "suggested that melanocyte dendrites might receive localized signaling from individual keratinocytes." Are there no vesicles in the cytosol of keratinocytes that are not juxtaposed to the melanocyte dendrite?

Response: Like in all cells, there are individual vesicles within the cytosol of keratinocytes. In this part of the text, we are highlighting the fact that at some cell-cell contact points there are pools of vesicles near the plasma membrane which is consistent with local exo/endocytic activity. However, we agree with the reviewer, in that, we should provide context for our claim and have revised the text to state that we observed individual vesicles throughout the keratinocyte but found pooled vesicles at some sites of contact with melanocytes. Please see lines 82-88.

Figure 3:

- Were the experiments measuring the calcium transients done in co-cultures in the presence of CaCl_2 ?

Response: All co-cultures were imaged in 1.06 mM CaCl_2 . The only exceptions were those where we explicitly tested for a role of extracellular calcium, as in figure 3, which is labeled with 0mM CaCl_2 . We have revised the text to be more specific about our experimental design and now specifically state that the imaging media (modified DPBS) contained 1.06mM CaCl_2 . Please see lines 95-98.

- In 3A, the co-cultures are presented based on the different donors. Is this an important consideration? Why were there 6 co-cultures from a particular donor and only 1 from another? Did the authors try mixing keratinocytes and melanocytes from different donors?

Response: We think it is important to note that we used different donors to show that our observations are universal to melanocyte – keratinocyte interactions and not specific to one donor or skin type.

All cultures were donor matched. In this study we did not mix melanocytes and keratinocytes from different donors. For ongoing projects, we have mixed them and we get the same results as when melanocytes and keratinocytes are from the same patient: significantly more co-culture melanocytes have Ca^{2+} transients and higher number of transients per cell than mono-cultured melanocytes. We show which data points came from which patient-derived cultures for transparency in our biological replicates.

- Why did the authors choose to examine the transients in only a 2.5-minute window? What happens if they look longer?

Response: To ensure that we were capturing the transients, we had to capture an image at a minimum of once every 250ms. To minimize photo bleaching of the GFP reporter and phototoxicity in the melanocytes, we limited the analysis to 600 frames - 2.5 minutes. To image for a longer period would have required less frequent images, which would have resulted in missing some of the calcium transients.

- Why was the data for whole cell and local transients pooled? This is confusing and unclear.

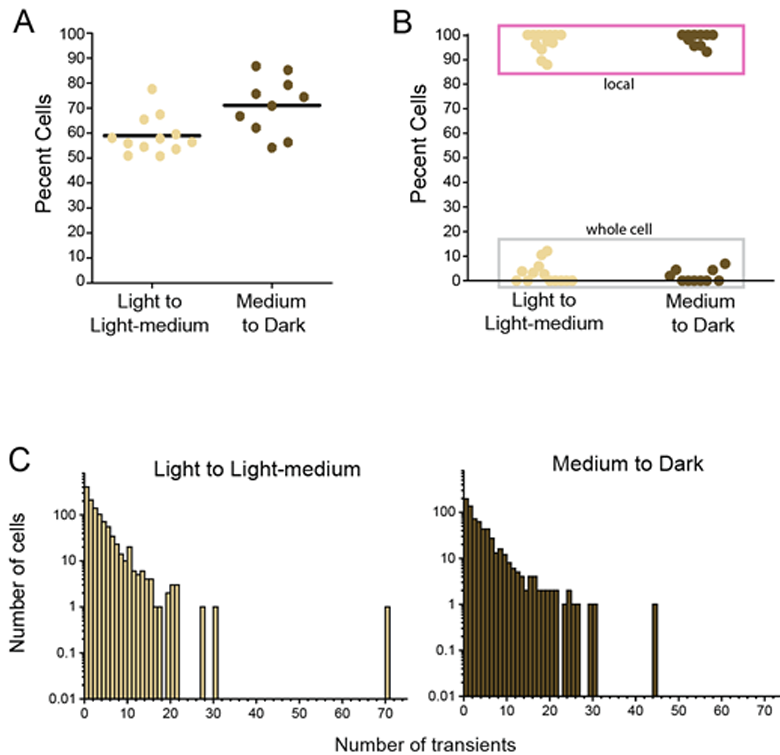
Response: We have revised the figure to separate the whole cell and local transients, instead of pooling them by donor. Please see revised Figure 3A.

- The authors claim that "The number of local dendritic transients detected during 2.5 minutes ranged from 0-72 per melanocyte (Fig. 3E)." However, based on the data from the graph, the vast majority of cells have between 0 and 10 transients, with anything more being quite uncommon.

Response: The reviewer is correct - the majority of cells with transients have 1-10 transients. However, it is also true that the range in number of transients per cell is 0-70 (we have revised the text to include the correct range which is 0-70 not 0-72; please see line 115). The text and figure are not inconsistent, and we believe both provide a full description of the results to the reader. However, we have revised the text to state the reviewer's point: "of the melanocytes with transients the majority had 1 – 10." Please see lines 115-116. In addition, we have made a few other changes to this figure panel. 1) We changed the graph to a \log_{10} scale which will make it easier to see the number of cells at higher x axis values. 2) In response to another comment below, we have displayed the number of transients per cell for both mono-cultured and co-cultured melanocytes. In order to compare the two data sets, we have normalized to the number of cells per condition and have presented the data as "percent cells". Please see revised Figure 3F.

- Panel E takes data from 27 co-cultures from 7 donors whereas panel A takes data from 22 co-cultures and 6 donors. The phototypes of the donors can be of importance this should be mentioned. Why the discrepancy? Are the 22 donors in 3A included in the 27 from 3E?

Response: Panel E contains data from panel A as well as data from cultures from another donor. We have revised the figure legend to state this. The reviewer brings up a good point about the phototype of donors. Since we used de-identified neonatal foreskin we did not have enough information to assess skin phototype via the Fitzpatrick scale (which includes information about eye color and how easily the person burns/tans). However, we did catalog the pigmentation status of the donors' skin in the broad categories of light, light-medium, medium, medium-dark, and dark. We used co-cultures derived from all 5 categories throughout the study. The phenomena we describe is consistent across melanocytes from different donors. We have gone back through the data in panel 3A and 3E and specifically looked at the difference between cultures derived from light to light medium pigmented skin and cultures derived from medium to dark pigmented skin. We observed that: 1) The average percent cells with transients is slightly higher in co-cultures from darker pigmented skin; 2) The distribution of the number of transients per cell is slightly different between lighter and darker pigmented skin. While both of these observations are statistically significant, they are within the same order of magnitude. Please see the graphs provided below: A) Percent of co-cultured melanocytes with one or more Ca^{2+} transients. Co-cultures derived from light to light -medium skin had fewer melanocytes with Ca^{2+} transients ($58.9\% \pm 7.4\%$ (mean, black line \pm s.d.)) than those from medium to dark skin ($71.1\% \pm 11.3\%$). Significance by Two Sample T-test p-value < 0.01. Data from Figure 3A separated by donor skin color. B) Percent of melanocytes with whole cell (WhC) Ca^{2+} transients or with local (L) Ca^{2+} transients from (A). C) Number of transients per cell was different between co-cultured melanocytes from lightly pigmented skin and darker pigmented skin (statistically significant by Mann-Whitney test ($U=339434.5$, $Z = -3.57$, p-value = 0.00035) Of the 64% co-cultured melanocytes with Ca^{2+} transients from lighter pigmented skin, 90% had between 1 and 8 transients. Of the 71% co-cultured melanocyte with Ca^{2+} transients from darker pigmented skin 90% had between 1 and 9 transients. Data from Figure 3E. Independent of skin color, melanocytes that were co-cultured with keratinocytes had a higher percentage of cells with calcium transients and more transients per cell than the melanocytes that were in mono-culture.



- Panel 3E also shows that approximately 600 out of 1793 melanocytes had 0 transients in the 2.5 min period. Is this consistent with the data from 3A?

Response: Yes. 1193 melanocytes have at least one transient, during the period we measured, which is 66.5% of melanocytes. Since very few cells have whole cell transients (< 10% total for any single donor). This is consistent with the range in panel 3A.

- Why were only 3 co-cultures from 3 skin donors analyzed for the analyses in 3F?

Response: The phenomena is consistent across cultures. This distribution is representative of the all donors. Therefore, we chose 3 cultures at random to quantify the spatial spread of individual transients.

Figure 4

- In panel 4B, at 3.25s, the signal appears to be less than at 2.75 and 3.75s. Why is this the case and why is the graph for 3.25s not presented in panel C?

Response: Intracellular Ca^{2+} does not always increase monotonically. This is similar to what is seen in neuronal dendrites. There was a small transient, and half a second later a much larger transient. As can be seen in Fig3B and Fig4F, there can be multiple transients within the same region of the dendrite. We did not include all time points in the graph because it made the graph too busy. However, the right panel 4C provides the temporal information for the entire imaging experiment at multiple regions of the dendrite, as indicated by the distance from the starting point (*).

- In panel 4C on the graph to the right, what are the authors trying to show?

Response: The graph for panel 4C shows the spatial distribution of calcium spread over time. The right panel shows the time course of the fluorescence signal (as a read out for Ca^{2+}) at the indicated locations on the dendrite. We have revised the figure legend to be clearer and have revised the figure to indicate that the x axis of this panel is time.

- Panel 4B presents data from 2.25 to 7.5 seconds while panel D presents from 10.25 to 24.95 seconds. Is there any significance to these different times or durations?

Response: We were imaging for windows of 2.5 minutes. We were not synchronizing activity at this point, so we are giving the time relative to the initiation of imaging. There is no significance to the time. As to the durations, they varied, which is why we showed both panels. In neurons there is also considerable variability of the duration of calcium spiking. The physiological significance of this variability has been the subject of many studies for 40 years but is still unresolved.

- The data from panel 4F is incomprehensible. What are the authors trying to show?

Response: We thank the reviewer for alerting us to this point of confusion. Panel 4F is a kymograph from a representative image of repeated Ca^{2+} transients in which the calcium intensity along a line through the dendrite is plotted over time (arrows) in panel 4D. We have revised Figure 4D,F and the corresponding legend to state that this is a kymograph from a line drawn along the dendrite in 4D. We have also revised the figure panel by indicating that the arrows point to repetitive Ca^{2+} transients.

- In the text, the authors claim that "In those dendrites that had multiple Ca^{2+} transients over time, the repeat transients initiated from the same region of the dendrite (Fig. 3F)." Do the authors mean 4F and not 3F?

Response: We thank the reviewer for catching this mistake. Yes, we meant 4F. We have made this correction (line 125).

Figure 5:

- What are unlabeled melanocytes?

Response: Unlabeled melanocytes are those that we have not transduced with GCaMP6f. We have revised the figure legend to state this. Please note that for clarity we have removed the origin panel A for figure 5. The graph showing the percent melanocytes with Ca^{2+} transients in different culture conditions is now figure 3H.

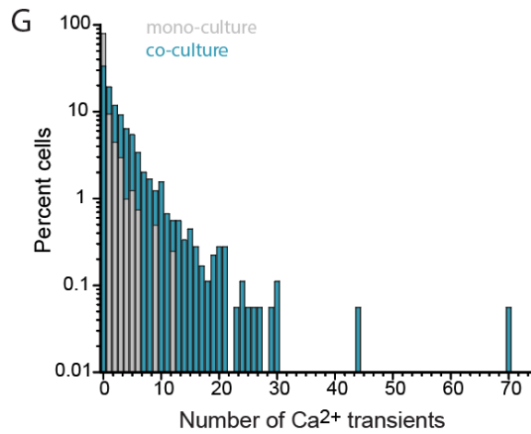
- Panel 5B gives the scale on the y-axis as % pre-treatment level. What does this mean? What is the pre-treatment? Treatment of CaCl_2 should increase the number of cells with transients, so this percentage should be higher than 100.

Response: 100% is the level of melanocytes before the treatment. Thus, 100% would indicate an equal number of melanocytes with Ca^{2+} transients before and after the treatment, as seen in normal imaging media (modified DPBS, 1.06mM CaCl_2). What we are plotting here is the number of melanocytes with Ca^{2+} transients during the treatment divided by the number before the treatment (pre-treatment). We have changed the y axis to be fold change relative to the melanocytes in regular imaging media (1.06mM CaCl_2).

For all treatments, the number of cells with Ca^{2+} transients are presented relative to the number of cells with Ca^{2+} transients observed in default imaging media (which has 1.06 mM CaCl_2). Thus, in panel 5B (now 5A) the 1.06mM CaCl_2 is the same CaCl_2 concentration as the pre-treatment. We have revised the text and figure to alleviate the confusion. Please see lines 129-135 and revised figure 5 with corresponding legend.

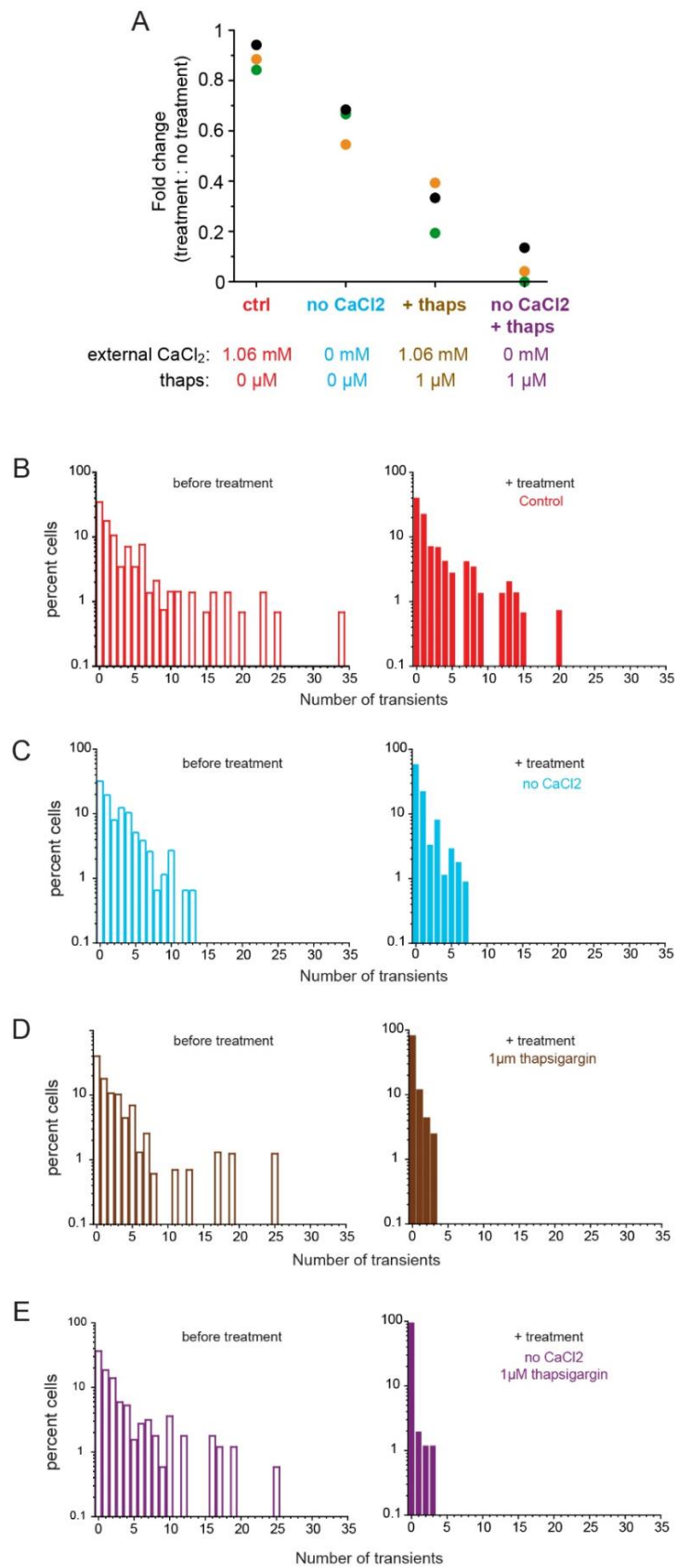
- As there are a great deal of results and they are not all clearly explained, what is the result when melanocytes are cultured alone in the presence of CaCl_2 ? How many calcium transients are there and how does this compare to the co-culture system?

Response: Few mono-cultured melanocyte (less than 20%) have Ca^{2+} transients. Of the mono-cultured melanocytes that do have transients, there are fewer overall number of transients per cell. To provide clarity on this matter we have first moved figure 5A to figure 3F. We have revised the text (lines 98-102, 109-117) and figure to be clearer about which conditions are co-cultures vs mono-cultures. Second, we have revised figure 3 to include data showing the number of transients per cell for both mono-cultured and co-cultured melanocytes (now Fig. 3G, see below). In addition, we have revised the text to include information regarding the number of transients per mono-cultured melanocyte (lines 109-117).



- Why are the scales different between panels C and D?

Response: We have changed the scales so they are the same and changed the y-axis to \log_{10} so it is easier to see the high x-axis data points. We have also taken panel 5C,D and split it into 4 panels so a direct side by side comparison can be made between baseline assessment of number of transits to the number of transients after treatment. Please see revised Figure 5 (below).



- What is the Control sample in panel 5D and where is the CaCl_2 1.06mM?

Response: The control sample in panel 5D is the 1.06mM CaCl₂ (no thapsigargin) from panel 5B. As discussed above we have revised the text and the figure to be clearer. Unless stated otherwise, all cultures were imaged in the presence of 1.06mM CaCl₂.

- Why is there such a considerable difference between the distribution of the blue bars from panels 5C and 5D? Again, the authors must be clear here. What is the pre-treatment and what is a treatment of 0 mM CaCl₂?

Response: The pre-treatment is the period in which the melanocyte are in normal imaging media (modified DPBS with 1.06 mM CaCl₂) before treatment. We have revised the figure so that the number of transients in the before treatment imaging is next to the number of transients after each treatment. Please see revised figure 5panels (C-F) where we have changed how we labels to before treatment and +treatment.

- The title of this figure is "External and internal Ca²⁺ pools contribute to melanocyte dendritic Ca²⁺ transients." Were the measurements taken exclusively from the dendritic transients?

Response: Yes. The measurements were taken from the dendritic transients. We have revised the figure legend to address this.

Figure 6 :

- The authors state that "the localized Ca²⁺ transients in melanocytes elicited by ET-1 (Figure 6A) resembled the spontaneous Ca²⁺ transients in co-cultures of melanocytes and keratinocytes without the addition of exogenous ET-1 (Fig. 4)." Could the authors please clarify where in Fig. 4 a graph like that in panel 6A is shown? Are they referring to Fig. 3? If so, where is the similarity?

Response: We thank the reviewer for pointing out the need for more information to back up our claim about the similarities. ET-1 elicited more Ca²⁺ transients within the melanocyte than those seen in the spontaneous Ca²⁺ transients. While the frequency of the transients was higher, the spatial distribution of the dendritic transients caused by ET-1 was similar to the spontaneous transients. We have provided more data on the physical distance of the spread of dendritic Ca²⁺ caused by the ET-1 and directly compare them to the spontaneous Ca²⁺ transients of the co-cultured melanocytes (revised Figure 6 panels C-E). Movie 3 (which is referenced in the text) provides a better visual of the ET-1 induced transients. We have kept the movie reference, removed the reference to Fig6A and add a reference for the new figure panels.

- Were the experiments with ET-1 done in the presence of CaCl₂?

Response: Yes – all of the ET-1 experiments were done in the presence of 1.06 mM CaCl₂. We again thank the reviewer for pointing out where we need to provide more information about the experimental set up.

Figure 7

- For both panels A and C: what is the pre-treatment? What is the control?

Response: "Pretreatment" for all figures is the cells in normal imaging media, which does not have growth factors or serum but includes CaCl₂. The measurement for the pretreatment, as in figure 5, is the number of melanocytes with Ca²⁺ transients in normal imaging media. What we are plotting is the number of Ca²⁺ transients after treatment with antagonist divided by the number of melanocytes with Ca²⁺ transients before treatment. We have revised Figure 7 so the y axis is defined at fold change. The control condition is the imaging media with the vehicle for the antagonist. We have revised the figure legend to include this information.

- The authors have not demonstrated that their antagonists work efficiently.

Response: We thank the reviewer for pointed out the need to provide evidence/discuss that the antagonist work efficiently. Supplemental Figure 2H and Supplemental Figure 3D show that both BQ788 and atropine inhibit the increase in Ca²⁺ transients induced by ET-1 or ACh in monocultured melanocytes. Kang et al., 1998 has previously characterized these antagonists in melanocytes. In ongoing projects outside of the work presented in this manuscript we have found that other secreted factors can induce localized dendritic Ca²⁺ transients in melanocytes providing further evidence that other factors besides ET-1 and ACh could be responsible for the remaining dendritic transients present after the treatment with BQ788 and atropine.

- In panel 7B, what is the level of knockdown for each siRNA?

Response: Due to an inability to find a good specific antibody, we have not been able to shown reduction of the receptors for endothelin at the protein level. However, we have been able to repeatedly demonstrate that dsRNA in the melanocytes against the EDNRB receptor significantly reduces the calcium spiking in the melanocytes but neither scrambled dsRNA nor dsRNA against EDNRA have any effect. We have also not been able to validate the knock down at the RNA level. None the less, the effect is robust and reproducible enough and the difference in the targets is, we believe, a strong internal control.

In our efforts to confirm knock down of these receptors in melanocytes, we have repeated the experiment multiple times using different donor cells – all with the same results: DsiRNA against EDNRB significantly reduces the number of melanocytes with Ca²⁺ transients compared to control, and there are no effects from DsiRNA against EDNRA and no effects from the non-target control. However, we have been unsuccessful at finding an antibody that can detect either receptor in our controls (non-targeted DsiRNA and no DsiRNA) via western blot (we have purchased multiple different antibodies). We have verified that our controls express EDNRB at the RNA level but do not see EDNRA expression at the RNA level. This is to be expected for EDNRA. Previous studies report little to no expression of this receptor in many patient derived melanocytes. Therefore, we know that melanocytes are expressing EDNRB and respond to ET-1 but we have not been able to show it at the protein level.

Regarding the KD using DsiRNA: We have not been able to get reliable detection of this particular RNA (with or without the DsiRNA) using qPCR even though we have tried numerous primers. However, we are confident that DsiRNA works in melanocytes - we have used the technique to KD tyrosinase in multiple biological replicates and have consistently shown significant decrease in tyrosinase expression (0 to 0.2 relative expression level compared to control). We also know that EDNRB DsiRNA is showing specificity because only the two EDNRB DsiRNA reduce the number of melanocytes with transients and not the non-target or EDNRA DsiRNA. Others have seen that, for some transcripts, siRNAs can inhibit translation but do not promote mRNA degradation. This is consistent with our observations. It is also possible that KD is most effective when melanocytes are co-cultured with keratinocytes. We have tried validating the KD in co-cultures and had the same issues as when we tried to validate on mono-cultured melanocytes.

We have added new data that show KD of EDN1 (ET-1) and choline acetyltransferase (effectively ACh) in keratinocytes significantly reduces the number of melanocytes with Ca²⁺ transients (Revised Figure 7C,D and 7F,G). Since this provides evidence that the ET-1 and ACh are keratinocyte derived we believe the findings stand without the EDNRB KD data. However, we would like your input about the inclusion of the DsiRNA data with KD validation.

- Knocking down of the endothelin receptors in the melanocytes does not show clearly that the keratinocytes are responsible for the ET-1 signal. In order to clearly demonstrate this, expression of the EDN1 gene must be silenced in keratinocytes and the absence of the protein must be confirmed. In this way, the role of keratinocyte ET-1 will be accurately assessed.

Response: We have done this additional experiment (lines, 160-163, revised Figure 7, panel C and D). The results confirm that KD of ET-1 in keratinocytes reduces the number of melanocytes with Ca²⁺ transients.

- A similar principle is true for the acetylcholine effect. Knockdown of (for example) choline acetyltransferase in keratinocytes would inhibit the production of the acetylcholine and therefore clearly indicate whether or not keratinocyte acetylcholine is important for the melanocyte Ca²⁺ transients.

Response: We have done this additional experiment (lines, 181-185, revised Figure 7, panel F and G). The results confirm that KD of choline acetyltransferase in keratinocytes, and thus production of ACh by keratinocytes, reduces the number of melanocytes with Ca²⁺ transients.

Figure 8

- What is the significance of the graph in panel 8C?

Response: Panel 8C provides the dimensions of individual spine-like structures which is important information for characterizing these structures. We have revised the figure legend to state that.

- The authors state that "some dendrites having no detectable spine-like structures," but this is not reflected in panel 8D.

Response: Panel 8D shows that on average 50% of the dendrites have clearly visible spines. This means that about 50% of dendrites do not have detectible spines, which is stated in the manuscript (line 196-198).

- Panel 8F does not show a clear interaction being made between the melanocytes and the keratinocytes. A confocal image showing overlap or a 3D reconstruction would be necessary.

Response: We have added a Z,XY image to panel 8F to better show the interaction between the keratinocyte and melanocyte highlighted in the figured.

Also, stronger way of resolving this is from our EM data that shows the keratinocyte processes interact with melanocyte spine-like structures (Fig. 10A, now revised to highlight keratinocyte processes and Sup Fig 4B) including a z-stack (SupFig4C) . We have revised Figure 2 to include color coding for melanocytes and keratinocytes (and corresponding keratinocyte processes) (Panels D and G). In addition we have included a 3D reconstruction of keratinocyte process (panel H) with the corresponding EM image (G) where we show a lower magnification view while highlighting the melanocyte and keratinocyte used in the 3D reconstruction.

- Do the dendrites interact with the keratinocyte envelope structures when there is re-shaping of the spines (panel 8H)?

Response: The keratinocytes were not labeled with a fluorescent protein in the cultures used for the imaging in panel 8H. Therefore, we cannot speak to whether or not keratinocyte processes were interacting with the spines as they were undergoing morphological change. However, Figure 2B shows that keratinocyte processes do interact with melanocyte dendrites that are undergoing morphological changes.

- Are the spine-like structures present on the dendrites of melanocytes when they are alone in culture?

Response: Some melanocytes do have spine-like structure on their dendrites when cultured alone. However, there much fewer on mono-cultured melanocyte dendrites.

- Panel 8F also shows that the keratinocyte envelope structures clearly do not wrap around the melanocyte dendrite. Perhaps a different word should be used to describe this phenomenon.

Response: In the description of Figure 8F, we did not say that this particular GFPmem-labeled keratinocyte wrapped around this particular melanocyte dendrite, nor did we say that all keratinocyte wrap around melanocyte dendrites. However, there are many cases of a keratinocyte with processes wrapped around a melanocyte dendrite, as shown in the figure 2A. In addition, Figure 2D-F and supplemental Figure 4C show that in intact skin keratinocytes wrap around melanocyte dendrites and spines.

- Do the keratinocyte membrane structures interact with the melanocyte spine-like structures?

Response: Yes, we see the keratinocyte membrane protrusions interact with the melanocyte spine-like structure. First, they in close proximity. Second, we observe them move together in unison at the apparent contact site. Panel 8G shows that keratinocyte plasma membrane interacts with the spine-like structures and Figure 10A, Supplemental Figure 4B,C show that keratinocyte processes interact with spine like structures in-situ.

Figure 9

- The kymographs in panel C are very hard to see and discern.

Response: We thank the reviewer for alerting us to this. We have made them larger.

- How many of the total dendritic transients were found in clear, spine-like structures?

Response: The quantification of the dendritic transients was done from data acquired using a 10x objective. At that resolution we can only resolve dendrites and not dendritic spines. To quantify the number of spines with Ca²⁺ transients from data acquired using a 60x objective. Alas, at this high a magnification, it is very difficult to catch events, which is why many more events are quantified on dendrites relative to dendritic spines.

- Are these small, unresolvable spine-like structures in close proximity/contact with keratinocytes?

Response: Since the keratinocytes in this figure were not labeled with a fluorescent reporter we cannot definitively claim that they are in close proximity. However, at the time of imaging all fields of view were visually inspected in infrared-DIC to ensure that the melanocytes were completely surrounded by keratinocytes. Thus, it is

highly probably that the unresolvable spine like structure in this figure was in close proximity/contact with the adjacent keratinocyte.

In contrast, in the EM data we can confidently say that all spines observed were in close proximity/contact with keratinocytes.

Figure 10

- The authors used the lack of keratin filaments as a marker for melanocytes, but this is not accurate, as melanocytes may express some keratins.

Response: We agree with the reviewer that “melanocytes may express some keratins”. However, the level of keratin in melanocytes is much less than that of keratinocytes which is reflected in the EM staining. We have revised the text to be more specific. Please see lines 208-209.

- Where are the spine-like structures in panel 10B?

Response: We have labeled the spine-like structures that are visible on the 3D model in revised Figure 10D

- Figure 10C needs to show the shape/size of the spine-like structures from melanocytes in culture in comparison to those found in the skin side-by-side for any meaningful comparison.

Response: We have revised Figure 10C to include the range in head diameter and total length for spines in co-cultures.

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

Belote et al studied change of Ca²⁺ transients in melanocyte dendrites driven by neighboring keratinocytes in a co-culture system. They showed that melanocytes dendrites and keratinocyte protrusions closely interact. Co-culture with keratinocyte promotes Ca²⁺ transients in melanocyte dendrites, which is enhanced by ET1 and ACTH, known to be produced by keratinocytes. While the study is carefully performed with large amount of data at a high resolution, providing good basis for a culture system to study melanocyte/keratinocyte signal transduction, there were several concerns such as inconsistent data, lack of data showing the keratinocyte/melanocyte interaction rely on dendrites/compartments, functional data and mechanistic view and similarity to neuron are weak.

Major concerns:

1) It seems unfair to compare Ca²⁺ transients in certain small areas of dendrites (Fig. 3B and Fig. 4) to whole cell transients (Fig. 3C) and conclude that spontaneous Ca²⁺ transients are compartmentalized mainly in dendrites. It makes more sense to compare Ca²⁺ transients in any areas of dendrites to those in similar size of areas in cell body to show enrichment of transients in dendrites but not cell body. There seems to be no data to show there is more local transients in dendrites than local transients in cell body.

Response: The reviewer brings up a good point regarding local Ca²⁺ transients in the cell body vs the dendrites. We observed very few cell body Ca²⁺ transients that were localized to just to the cell body. The overwhelming majority of localized transients were in the dendrites. We have revised the text (lines 100-102) and figure 3 (panels C and D) to include this data and address this point.

2) SFig.1 showed that in both melanocyte/keratinocyte co-culture and melanocyte single culture, there is no difference in the Ca²⁺ transients in between keratinocyte culture medium (do not contain ET-1) and melanocyte culture medium (contain final concentration of 10nM ET1). However, addition of 10nM ET1 in co-culture (Fig. 6) in keratinocyte culture medium and melanocyte single culture (SFig.2) can induce more robust Ca²⁺ transients. These data seem inconsistent about the role of ET-1 in inducing more Ca²⁺ transients.

Response: We thank the reviewer for pointing out that we need to be clearer in the text regarding our imaging conditions. As discussed in the response to reviewer 1 above, all cultures for these experiments were imaged in imaging media (modified DPBS, 1.06mM CaCl₂, with no additional growth factors or serum) and washed 3 times before imaging. The only exceptions were those experiments in which we explicitly added ET-1, and that is indicated in the text/figure. In Figure 3 we are comparing cultures that were *grown* under different conditions (different media

or cell types present) but all cultures were imaged in the imaging media which contained no additional growth factors. We have revised the manuscript accordingly (lines 95-98, 107-108, 448-452, figure legends for Fig. 3 and Supplemental Fig. 2 - Supp Fig 2A is now a schematic of the experimental design). We believe that with this information the concern from reviewer 2 is no longer an issue.

3) Fig. 6A showed that with addition of ET-1, there is also quite obvious increased Ca^{2+} transients in cell body. Does ET-1 induce more cell body Ca^{2+} transients than baseline and ACTH?

Response: We have not assessed the effects of exogenous ET-1 and ACh on the number of whole cell/cell body Ca^{2+} transients in melanocytes. Since this manuscript is about local Ca^{2+} transients in melanocyte dendrites due to endogenous secreted factors, we think this analysis is tangential to main point of the paper and thus outside the scope of the manuscript.

4) It wasn't clear whether melanocyte Ca^{2+} transients rely on the contact of their dendrites with keratinocytes, or keratinocyte secreted factors, or both. If both, which is more important/required? It was already known that keratinocyte secreted factors can induce change in melanocyte Ca^{2+} transients, so it is more important to show stronger data that the direct dendrite contact is important. SFig.1 seems to show some data for this (culture of melanocytes with keratinocytes conditioned medium), but it is difficult to interpret because there is very few description of this experiment.

Response: Our data demonstrates that the Ca^{2+} transients in melanocytes rely on factors that are secreted by neighboring keratinocytes. So both are important: secretion of the factors as well as immediate proximity to the cell that is secreting the factor. The conclusion that both are important is based on the observations that:

- We can elicit local Ca^{2+} transients in melanocyte dendrites by filling the bath with exogenous ET-1 and ACh.
- Also, we can reduce Ca^{2+} transients in melanocyte dendrites when co-cultured in direct contact with keratinocytes by blocking ET-1B and acetylcholine receptors
- We can reduce calcium transients in melanocytes by reducing synthesis of the ET-1 or ACh in keratinocytes.
- They cannot be elicited when the keratinocytes are in the culture but physically separated from the melanocytes.

Together this shows that factors secreted by the keratinocytes can elicit Ca^{2+} transients in melanocyte dendrites, but that keratinocytes need to be in close proximity to the melanocytes. Our conclusion is that the concentration of these secreted factors only reaches a physiologically significant level at the receptors on the surface of the melanocytes when the two cells are in direct contact. Thus, both contact and secretion are both required.

5) There is no mechanism about why the contact between melanocyte dendrite and keratinocyte is important for Ca^{2+} transients in melanocytes. Also no mechanism why more transients were observed in dendrites than cell body. Is there evidence that more ET1 and ACTH receptors are located in the dendrites?

Response: As described in the response to comment #4 we conclude that local secretion from keratinocytes provides enough ET-1 or ACh to produce a response in melanocyte dendrites. We have seen pools of vesicles in the keratinocyte cytosol in close proximity to melanocytes dendrites (Fig 2E, Fig. 10D, and Supplemental Fig. 5C). It is possible that keratinocytes secrete ET-1 and ACh at sites along the dendrite but not the cell body. However, we have not conducted a comprehensive study on the localization of pooled vesicles in keratinocytes in situ and can only anecdotally say that we have not observed pooled vesicles at the keratinocyte plasma membrane at sites adjacent to the melanocyte cell body, nor have we quantified the contacts between the keratinocytes and cell body of the melanocytes. Regarding melanocyte ET-1 and ACh receptor localization: We have not conducted this experiment. In part because we have not been able to find an antibody that is of sufficient quality to detect endogenous EDNRB on melanocyte in co-culture with keratinocytes and because there are 5 different muscarine ACh receptors.

6) The whole manuscript showed "compartmentalized Ca^{2+} transients" but did not show whether this elicits local changes but not global changes in melanocytes. Most known function of ET1 and ACTH in melanocytes are through changing transcription of several melanocytic genes, which needs to go to nucleus and cannot be a local change, even if the signals were received locally. Without such data, it is difficult to conclude that melanocyte dendrites mimic neuron in compartmentalized response to signals.

The authors mentioned the similarity of melanocytes to neurons in their dendrites properties and functions many times

in the text, but there is no neuron data as control.

Response: We agree that most of the studies on the effects of ET-1 and ACh on melanocytes have looked at global changes such as proliferation or transcription. However, it has been clearly demonstrated that secreted factors can induce both local and global changes (this is true for neurons as well). ET-1 stimulation of melanocytes is followed by PKC translocation and phosphorylation of numerous proteins (Imokawa, G. et al, *Pigment Cell Research* (1997) 10:4, 218-228). This occurs on the timescale in which we are imaging Ca^{2+} transients. In addition, ET-1 can also induce cAMP signalling which is known to directly activate the rate limiting melanin synthesis enzyme tyrosinase which can lead to local increase in pigment production. We believe that it is important to note the difference in time scale for our studies compared to other studies that look at the effects of these factors on the order of days not minutes. Please see our revised discussion (lines 284-294)

In addition, we have revised the manuscript so all comparisons of melanocyte dendrites and spine-like structures to neuronal/glia counter parts are in the discussion and not in the introduction and results. While we do not want to overstate our claims, we believe that there is sufficient data to discuss the parallel to Ca^{2+} transients in neurons: 1) the dendritic transients in melanocytes have the same characteristics of IP3 mediated dendritic transients in Purkinje neurons (we have referenced this in lines 271-274) and 2) the spine-like structures have similar dimensions, morphology and ability to spatially restrict Ca^{2+} transients. We have separated the data and speculation and have revised the manuscript accordingly. The introductory paragraph on dendritic processes in neurons and glia has been moved to the discussion: lines 251-262. The comparison of melanocyte spine-like structures to neuronal dendritic spines have been moved to the discussion: lines 305-307.

7) Spine structures on dendrites were studied in 1/3 of the paper, but it is not clear what is the importance/difference of this structure compared to dendrites and filopodia on dendrites in Ca^{2+} transients or other functions. Do filopodia also have Ca^{2+} transients? How about interaction of filopodia with keratinocytes?

Response: Our decision to include information regarding melanocyte filopodia was to show that the spine-like structures were different than previously reported filopodia on melanocyte dendrites. We have not done a full analysis of Ca^{2+} fluctuations in these structures, but can say anecdotally that we have observed a few Ca^{2+} transients in melanocyte filopodia. In co-cultures where melanocyte and keratinocyte plasma membranes were labeled with fluorescent proteins, we were able to see some filopodia interact with keratinocytes. However, the majority of filopodia on the melanocyte dendrites, like filopodia on neuronal dendrites, were very mobile with movements on the order of milliseconds and seconds, unlike the spines which were on the order of minutes to potentially hours. The sporadic events and mobility issue make the filopodia inaccessible to the kinds of quantification that could provide further insight at this time. In addition, since the focus of this project is on cell-cell communication and filopodia are involved in many cellular processes (not just cell-cell signaling), we believe that a full characterization of the filopodia is outside the scope of this paper and detracts from the main discoveries.

Minor concerns:

In Fig. 1C, it seems that many keratinocytes were not labeled by GFP. What percentages of melanocytes and keratinocytes were labeled by fluorescent proteins in this culture system?

Response: To facilitate imaging the extent of individual cells, we specifically tagged a subset of either the melanocytes or keratinocytes. This mosaic approach is necessary to be able to follow the processes from individual cells. When all of the cells are labeled the field is a flood of fluorescence where it is not possible to resolve distinct individual processes. We established the system such that approximately 30-60% of keratinocytes were labeled and 60-90% of melanocytes were labeled.

What is the cause of baseline Ca^{2+} transients studied in Fig. 3 and 4? Is it caused by certain supplements in the keratinocyte culture medium, such as BPE, Igf1 and EGF?

Response: As discussed above, we have revised the text and figure legend to be clearer about our imaging conditions. All cultures were imaged in a modified DPBS solution (DPBS, Hepes, glucose, 1.06mM CaCl_2 , 0.5mM MgCl_2 and glycine – no growth factors or serum). Cultures were washed 3 times in this imaging media and then the imaging experiment

was performed. The only time we altered the imaging media was when we added various drugs (thapsigargin, antagonist, and agonist) or removed the CaCl_2 to assess external Ca^{2+} stores.

Since we are imaging in a solution that contains no growth factors, we conclude that the source of the spontaneous Ca^{2+} are intrinsic to the cells within the co-culture. We show in Fig 6 and Fig 7 that we can reduce the number of cells with Ca^{2+} transients, and number of transients per cell, by blocking endothelin receptors or by blocking ACh receptors. Thus, we conclude that secreted endothelin and ACh are responsible for some of the spontaneous transients. The fact that we cannot eliminate all of the Ca^{2+} transients suggests that there are likely other secreted factors that also contribute (which we have found to be true in other ongoing projects)

How many layers of keratinocytes were in the culture system and is it comparable to human? In Fig. 1F, 3D image or Z-stack images showing each layer should be added to help the interpretation of data. What is the distribution of melanocytes in multiple layers of keratinocytes in the culture system? Are they mainly located in the basal layer as in human?

Response: We had 2-3 layers of cells in our co-culture. We have revised the text (lines 58-60) to be clearer about how many layers of keratinocytes are present in the co-culture. The bottom layer is composed of K14 positive keratinocytes and the top layers are K10 positive keratinocytes. Melanocytes reside in the bottom layer with dendrites extending throughout the different layers of cells. Please see new Figure 1G. Human skin has more layers than our co-culture system, which makes sense given that the co-culture system is not a full skin reconstruct.

Fig. 1A, how can TRP1 and cKit both in green?

Response: We wanted to provide a complete labeling of the melanocyte. As described in our response to reviewer 1, we used both mouse anti-cKit for plasma membrane and mouse anti-TRP1 for melanosomes. One secondary antibody (anti-mouse IgG) was used to detect both primary antibodies. This allowed a more comprehensive coverage of the processes of the melanocytes.

In Fig. 2D and E, it is difficult to appreciate how keratinocyte processes envelope dendrites of melanocytes. 3D reconstruction of TEM images acquired on serial sections (or something like SFig. 4) may be needed. It is also not clear based on what evidence the keratinocyte processes and melanocyte dendrites were identified on the TEM images, which may be helped by showing lower magnification images of the same area showing more defining details of both cell types.

Response: We thank the reviewer for pointing out the need for clarification on this matter. We have revised Figure 2 to include color coding for melanocytes and keratinocytes (and corresponding keratinocyte processes) (Panels D and G). In addition we have included a 3D reconstruction of keratinocyte process (panel H) with the corresponding EM image (G) where we show a lower magnification view while highlighting the melanocyte and keratinocyte used in the 3D reconstruction.

Figure number should follow the order they appear in the text. For example, Fig 3 and 4 were quite mixed in their order in text.

Response: We have revised the text and figures so that figure numbers in the text follow the order they appear in the figures.

October 11, 2019

RE: JCB Manuscript #201902014R

Dr. Sanford M Simon
Rockefeller University
Cellular Biophysics
1230 York Avenue
New York, New York 10065

Dear Dr. Simon,

Thank you for submitting your revised manuscript entitled "Ca²⁺ transients in melanocyte dendrites and dendritic spine-like structures evoked by cell-to-cell signaling". You will see that both original reviewers are now supportive of publication. Thank you for your efforts to revise the work and thank you for following up on the revision proposal and updates we had discussed since the paper was first reviewed at the journal. 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.

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- **Please include such a summary on the title page of the resubmission. It should start with "Belote and Simon..." to match our preferred style.**
- 2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2CH (if possible), 2A (right, magnifications), 8F (bottom mags), 10A (left, main image), S1D (magnifications), S5B (main micro)
- 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 indicate n/sample size/how many experiments the data are representative of: 4CE, 6DE, 7B, 8E, 9C, S2C
- 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 in the text for readers who may not have access to referenced manuscripts.
- Please include the species for all antibodies.
- Please include the sequences for all siRNA oligos used, including negative controls (if sequences were made available to you from the manufacturer).
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and processing of images:

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- b. Type, magnification, and numerical aperture of the objective lenses
- c. Temperature
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- 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.).

5) A summary paragraph of all supplemental material (including videos) should appear at the end of the Materials and methods section.

- Please include ~1 brief descriptive sentence per item.

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Sincerely,

Cédric Blanpain, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors improved dramatically their manuscript as recommended. They clarified key points and performed important experiments. As such, I am fully convinced by their works and major conclusions.

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

The authors sufficiently addressed comments from the previous review to be ready for publication.