RAS-mediated suppression of PAR3 and its effects on SCC initiation and tissue architecture occur independently of hyperplasia

Ji Ling, Maria Sckaff, Manisha Tiwari, Yifang Chen, Jingting Li, Jackson Jones and George L. Sen

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Original submission

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MS TITLE: RAS-dependent suppression of PAR3 and its effects on SCC tumor initiation and tissue architecture

AUTHORS: Ji Ling, Maria Sckaff, Manisha Tiwari, Yifang Chen, Jingting Li, Jackson Jones, and George Sen
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers share enthusiasm for the study and its conclusions. However, they raise a number of substantial criticisms that prevent me from accepting the paper at this stage. These critiques focus on a need for further quantification of the phenotypes, a need to gain a deeper insight into the residual hyperplasia phenotype, the protein levels of EMT markers (see Rev 2), improved citation and discussion of prior work in the field, consideration of how Par-3 isoform could affect interpretation, and questions about interpretation of spindle orientation vs. cell fate. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

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

Reviewer 1

Advance summary and potential significance to field

The work by Ling et al. describe the surprising finding that cell polarity drives tissue architecture but not hyperplasia. The authors show in a clinically-relevant human epidermal model that PAR3 disruption occurs during RAS/CDK4-driven SCC initiation and progression and that overexpression of PAR3 can rescue the disorganized tissue and spindle orientation defects, but does not rescue tissue hyperplasia. The RAS pathway drives both tissue disorganization through suppression of PAR3, as well as tissue hyperplasia through a PAR3-independent mechanism. This work will be of broad interest to the cancer and skin fields, and suggests that cell polarity and spindle orientation does not significantly impact tumor growth, which is wildly surprising, but may be responsible for metastasis in advanced tumors as tissue architecture disintegrates.

Comments for the author

Minor comments for revision

1) Is there a quantifiable way to reflect differences in tissue architecture between LACZ versus PAR3 overexpression? The epidermal hyperplasia looks morphologically distinct between the two conditions, with PAR3 OE almost resembling psoriatic skin.

2) Discussion of the authors findings with Mescher et al. 2017, J Exp Med, which found that epidermal loss of Par3 in mouse skin results in melanocyte hyperplasia and Vorhagen et al. 2018, Oncogene, which found that loss of Par3 in mouse skin decreased DMBA/TPA-mediated SCC progression but increased invasiveness.

3) A more active title for the paper that describes PAR3's role in tissue architecture independent from hyperplasia would make the paper's significance more clear.

Reviewer 2

Advance summary and potential significance to field

General comments:
This is a well designed and well executed study that makes good use of 3-dimensional organotypic skin models, which maximizes physiologic relevance. I have little to criticize with regard to the technical execution of the work. The data clearly establish a functional role for PAR3 in the early stages of of Ras driven cutaneous neoplasia, which models human skin SCC.

This work will be of interest to a wide range of epithelial biologists. With minor revision, this work would seem appropriate for JCS.

Comments for the author

Additional specific points:
1. While not absolutely essential, it would be very helpful for the field to know the degree to which PAR3 depletion and altered division angles studied in this paper correlate with PAR3 protein and cellular division angles in spontaneous epidermal SCC in people, and whether this correlates with tumor stage acros the SCC continuum (pre-cancerous actinic ketatosis, SCCIS, early vs late invasion.

Fresh, frozen, and FFPE tissues for this should be readily available. If there is a technical reason for why this study is not possible, could the authors please explain the limitation?

2. Authors may wish to speculate on the mechanism(s) by which Ras activation leads to PAR3 protein loss.

3. Authors note that while PAR3 and E-Cadherin proteins are depleted following Ras activation, the mRNA does not change significantly. That is a perfectly fine and interesting observation. However, in the same section, authors then state that because mRNA levels of EMT markers such as TWIST do not change, that EMT is not a feature of the SCC phenotype in the model. What about protein levels of those EMT markers?

Reviewer 3

Advance summary and potential significance to field

In this very interesting paper the authors have addressed the initial cellular changes that occur during tumorigenesis using an inducible SSC human skin xenograft model and address how these changes occur.
The authors show that SSC induction in their model system results in hyperthickening and an altered organization of the epidermal architecture that resemble SCC. Moreover, their results indicate a pathway in which RAS-dependent suppression of the polarity protein PAR3 results in loss of E-cadherin from cell-cell contacts leading to increased misoriented spindle orientation. Finally, they link this to Erk as pharmacological inhibition of MAPK pathway downstream of RAS activation rescued Par3 and E-cadherin localization, and mitotic spindle orientation, even though, interestingly, it could not reverse the hyperthickening phenotype.
The strength of this paper is the human organotypic engraftment model in combination with the tamoxifen inducible expression of Ras allowing to address and unravel early processes involved in human SCC tumor initiation). Moreover, their inducible model system allows the study of tumorigenesis in a physiologically relevant 3D in vivo environment (mouse) and is likely to mimic more closely human SSC.
Less strong aspects are that everything is based on immunofluorescent data without taking protein expression into account, much of the molecular mechanisms are not entirely new (not always acknowledged through references), albeit not yet reported within a human SCC-like setting. Their data is also poorly linked to other in vivo studies that addressed how Par3 control skin tumorigenesis. Perhaps most importantly, while the authors present intriguing links between Ras/Erk, Par3, E-cadherin and spindle orientation, how RAS and Erk regulate regulate Par3 expression, and whether loss of E-cadherin is essential in driving the Par3-dependent alterations in spindle orientation is not directly shown. In this light, it is surprising to not see any discussion to the in vivo studies on Par3 in mouse skin carcinogenesis, as in these papers (Iden et al Cancer Cell 2012; Vorhagen et al, Cancer Research, 2018) links between Par3 and Erk were established and Par3 distribution in human SCC was analyzed. In addition, Par3 in other systems clearly can regulate spindle orientation independently of E-cadherin, e.g. by controlling Lgn localization. This again is not discussed at all.
Finally, although U0126 treatment rescue spindle orientation and E-cadherin localization, hyperthickening and altered tissue organization (still forming these ridges moving into the dermis) still occurred leaving the question open how relevant the loss of Par3 and its effect on spindle orientation are for the progression of SSC. Having said that, this is an interesting contribution and much of those points are not easy to address and beyond the scope of the paper but could be better discussed and/or acknowledged.

Comments for the author

In addition to the general comments above, the following points should be addressed.
- Most of the essential data is shown in a qualitative manner and not based on proper quantification. The authors make a strong point on loss of Par3 and E-cadherin but all based on showing one fluorescent image.
It is absolutely essential that these datasets are properly quantified, also when rescued upon overexpression of Par3 or inhibition of Erk in the different figures.

Similarly, the authors make quite strong statements that overexpression of Par3, loss of E-cadherin or loss of
hyperproliferation but most of this data is based on hyperthickening. Although suggestive, hyperthickening can also arise due to altered cell death rates or slower transit times of keratinocytes. In fact the images shown for KI67 but not quantified as far as one can judge, suggest not so much change in KI67 when activating Ras, which seems surprising in light of ther studies. If the authors want to make strong statements that hyperproliferation is not affected it is essential that they properly quantify proliferation in their system also as it is a very interesting and important point.

- the authors make statements on par3 and E-cadherin loss based on IF. However, that technique really is about localization and much less useful in terms of loss of protein expression. As Par3 may have different roles in skin cancer development depending on whether it is at the membrane or not (see e.g. Iden et al Cancer Cell 2012), showing that their mechanism relates to a difference in protein expression is an important point. Do the authors see loss of Par3 and E-cadherin protein expression by western blot as well? Does inhibition of Erk rescues Par3 and E-cadherin protein expression?
In addition, it seems that the Par3 overexpression does not restore Par3 localization to control levels but instead seems to be highly overexpressed. Western blot analysis helps in documenting the extend of the overexpression.

- Although the loss of E-cadherin data are suggestive of E-cadherin being an important player downstream of Par3, it does not proof directly that loss of E-cadherin is essential to drive the Ras dependent phenotypes. Is overexpression of E-cadherin sufficient to rescue spindle orientation similar to Par3?

- Which Par3 isoform was used for overexpression? Par3 comes in different isoforms due to splicing and potential usage of alternative start codons, the main ones in the epidermis being 180kD, 150 kD and 100 kD and not all of these isoforms can interact with aPKC, which is known to cooperate with Par3 in driving Ras dependent papilloma formation.

- The authors refer to symmetric and asymmetric cell division but although not well acknowledged in the skin field, these definitions are coupled to fate. What the authors assess is spindle orientation, an important process but as the authors do not follow the fate of cells with random spindle orientation there is no direct outcome of fate. Excessive planar spindle orientation which are thought to drive self-renewal to increase the stem cell-like pool of cells has been linked to cancer. In fact, based on the hyperthickening data, in their model spindle orientation seems not to be connected to cell fate but rather random spindles is linked to a disrupted tissue organization observed in this cancer model. For these reasons, it is better to avoid using the terms SCD and ACD which are always connected to cell fate and instead use planar, random or perpendicular spindle orientation as that is what is really assessed by the authors.

- It was a bit surprising not to see any discussion or reference to the mouse skin cancer models on loss of Par3 and its dual role in keratoacanthoma and papilloma formation as these papers have explored Par3/aPKC expression in SCC (not altered), explored connections between Par3 and Erk, and provided evidence that loss of Par3 and aPKC promotes invasion. Similarly, the Williams group recently showed that loss of AJs controls spindle orientation (Lough et al, Elife 2019) but again this paper is not cited.

- The authors refer to cell adhesion changes downstream of Par3 and Erk, however, changes in cell-cell adhesion have not been directly addressed. The reduced E-cadherin localization at junctions is suggestive of a loss of adherens junctions, but this loss coiuld be compensated by other adhesion molecues, also as no obvious acantholysis or micro cel-cell blisters are observed. This should be rephrased in the abstract and in other places.

- Figure3C: is there excessive differentiation upon Par3 overexpression?

- Spindle orientation measurements: How many spindles were quantified in the different spindle orientation.

For Figure 3F, at least 3 animals should be used, however, the authors state 2-5 animals. For which groups were only 2 animals used?

- How does tamoxifen affect control tissues that do not express the Ras ocongene? As tamoxifen can affect in mice hair cycle, stem cell activation and proliferation and tissue organization, this is an important control.
Vehicle control is not tamoxifen treated CDk4 only 3dOrganotypic transplanted, correct?

- Quality of images. In general quality of several images could be improved.
For the first figure it would be good to insert a high magnification Figure 5: Is upon U01256 Par3 restored on IF? Image not so clear, is it mainly restored in the basal layer?
Figure 4 last panel Col7 can not be observed in the basement membrane in the controls.
Figure 2A: DAPI exposure seems different between controls and TMX treated conditions.

First revision

Author response to reviewers' comments

We thank all the reviewers for their insightful suggestions and comments which, we believe, have made this manuscript substantially stronger. Please see below for a point-by-point response to each reviewer's question. New texts written in the manuscript as a response to reviewer questions are underlined.

Reviewer 1:
Minor comments for revision
1) Is there a quantifiable way to reflect differences in tissue architecture between LACZ versus PAR3 overexpression? The epidermal hyperplasia looks morphologically distinct between the two conditions, with PAR3 OE almost resembling psoriatic skin.

We thank the reviewer for this question. We tried to find ways to quantify the tissue architecture between LACZ and PAR3 overexpression tissue but were not able to find a reasonable way to do it.

2) Discussion of the authors findings with Mescher et al. 2017, J Exp Med, which found that epidermal loss of Par3 in mouse skin results in melanocyte hyperplasia and Vorhagen et al. 2018, Oncogene, which found that loss of Par3 in mouse skin decreased DMBA/TPA-mediated SCC progression but increased invasiveness.

We have now discussed the above references in the discussion section as the reviewer suggested.

3) A more active title for the paper that describes PAR3's role in tissue architecture independent from hyperplasia would make the paper's significance more clear.

We have revised the title to reflect this. It now reads, "RAS-dependent suppression of PAR3 and its effects on SCC tumor initiation and tissue architecture occurs independently of hyperplasia."

Reviewer 2:
1) While not absolutely essential, it would be very helpful for the field to know the degree to which PAR3 depletion and altered division angles studied in this paper correlate with PAR3 protein and cellular division angles in spontaneous epidermal SCC in people, and whether this correlates with tumor stage acros the SCC continuum (pre-cancerous actinic ketatosis, SCCIS, early vs late invasion. Fresh, frozen, and FFPE tissues for this should be readily available. If there is a technical reason for why this study is not possible, could the authors please explain the limitation?

We thank the reviewer for this suggestion but we believe this is beyond the scope of the paper.

While pre-cancerous actinic ketatosis, SCCIS, and early vs late invasion tissue can be obtained, it would be extremely difficult to perform the study that the reviewer suggested. This type of study would be an entire manuscript on its own due to the following issues:
1. The tissue would first have to be screened to determine which patients had activated RAS pathway components (This could include oncogenic RAS, or RAF, MAPK/ERK activation, etc…).
2. The tissue would have to be co-stained for PAR3 expression and PH3 at the same time which would make it difficult to use paraffin embedded sections.
3. The tissue would then have to be stained with PH3 and each division angle counted and quantified. Although these would be coming from cancerous tissue, it is still difficult capturing cells at the right stage of mitosis. Because of the intense labor involved in performing all these cell division angle counts, the current paper under consideration has taken us 5 years to complete.

We also wrote in the Discussion section (final paragraph) that the next step is to follow up in human patient samples. It is written as, "It will be important in the future to determine the extent to which PAR3 loss leads to altered division angles and disrupted tissue architecture in spontaneous human SCCs. It will also be interesting to determine whether this also correlates with the progression of the tumor from pre-cancerous to invasion."

2. Authors may wish to speculate on the mechanism(s) by which Ras activation leads to PAR3 protein loss.

We have now done this in the discussion section. It is written as, "Our results demonstrate that activation of the MAPK pathway regulates PAR3 expression/localization to cell junctions however it is not clear the precise mechanism of how this is done. A previous study has shown that ERK2 directly interacts and phosphorylates Par3 at Ser-1116 in neurons (Funahashi et al., 2013). This results in the accumulation of Par3 at axonal tips. ERK has also been shown to be necessary for the proteasome dependent degradation of proteins involved in promoting cell cycle and migration through phosphorylation of its target proteins (Deschenes-Simard et al., 2013). Thus, it is possible that ERK2 phosphorylates PAR3 in our tumor model to causes its degradation."

3. Authors note that while PAR3 and E-Cadherin proteins are depleted following Ras activation, the mRNA does not change significantly. That is a perfectly fine and interesting observation. However, in the same section, authors then state that because mRNA levels of EMT markers such as TWIST do not change, that EMT is not a feature of the SCC phenotype in the model. What about protein levels of those EMT markers?

We have now performed the experiment and found that there is no protein expression of SNAIL or SLUG in either VEH or TMX treated CDK4/RAS epidermis (Supplementary Figure 1F). This suggests that our tumor model reflects the early stages of tumorigenesis prior to EMT. NIH 3T3 cells were used as a positive control for SNAIL and SLUG protein expression (Supplementary Figure 1F).

Reviewer 3:
1) The authors make quite strong statements that overexpression of Par3, loss of E-cadherin or loss of hyperproliferation but most of this data is based on hyperthickening. Although suggestive, hyperthickening can also arise due to altered cell death rates or slower transit times of keratinocytes. In fact the images shown for KI67 but not quantified as far as one can judge, suggest not so much change in KI67 when activating Ras, which seems surprising in light of ther studies. If the authors want to make strong statements that hyperproliferation is not affected it is essential that they properly quantify proliferation in their system, also as it is a very interesting and important point.

We thank the reviewer for this suggestion. We have now quantified all of our KI67 results. Our data suggests that proliferation increases after RAS activation (Supplementary Figure 1B-C) as expected. However overexpression of PAR3 in TMX day 20 grafts does not change the Ki67 positive percentage as compared to the LACZ control samples (Figure 2F). This suggests that PAR3 overexpression can rescue the cell division mis-orientation (Figure 3D-E), ECAD expression (Figure

2G, Supplementary Figure 2A), CLDN1 expression (Figure 2H) but not proliferation (Figure 2F). Similarly, knockdown of ECAD at TMX day 5 led to mis-orientation of cell division angles (Figure 4F-G) at an earlier stage of tumor initiation but had no impacts on the percentage of Ki67 positive cells (Figure 4D-E). These data suggest that the impacts of RAS on proliferation and tissue organization/cell division orientation can be decoupled.

2) The authors make statements on par3 and E-cadherin loss based on IF. However, that technique really is about localization and much less useful in terms of loss of protein expression. As Par3 may have different roles in skin cancer development depending on whether it is at the membrane or not (see e.g. Iden et al, Cancer Cell 2012), showing that their mechanism relates to a difference in protein expression is an important point. Do the authors see loss of Par3 and E-cadherin protein expression by western blot as well? Does inhibition of Erk rescues Par3 and E-cadherin protein expression?

We have now provided the Western blot results as well as quantitation of it. In the new Figure 1F-1G, RAS activation for 10 and 20 days (TMX day 10 and TMX day 20, respectively) leads to the progressive and dramatic loss of both ECAD and PAR3 on the protein level as compared to the VEH day 20 (no tamoxifen) samples. Furthermore, PAR3 overexpression in CDK4/RAS epidermis restored the expression of ECAD on the protein level as compared to the LACZ/CDK4/RAS epidermis treated with Tamoxifen for 20 days (Supplementary Figure 2A). Lastly, inhibition of ERK with U0126 rescues PAR3 and ECAD protein expression (Figure 5F).

3) In addition, it seems that the Par3 overexpression does not restore Par3 localization to control levels but instead seems to be highly overexpressed. Western blot analysis helps in documenting the extend of the overexpression.

We have now shown this by Western blot and quantitation of it. This is now shown in Supplementary Figure 2A. Notably overexpression of PAR3 also rescued the expression of ECAD (Supplementary Figure 2A).

4) Although the loss of E-cadherin data are suggestive of E-cadherin being an important player downstream of Par3, it does not proof directly that loss of E-cadherin is essential to drive the Ras dependent phenotypes. Is overexpression of E-cadherin sufficient to rescue spindle orientation similar to Par3?

This is an important and interesting question but we feel that it is beyond the scope of the current work for the following reasons:

1) We have not cloned E-cadherin into a retroviral construct and thus do not know what level of expression if any would be produced inside regenerated human tissue.
2) It is not clear whether E-Cadherin would even localize to the right place and thus may not rescue just for those reasons.
3) Overexpression of E-Cadherin may lead to off-target effects or other impacts that obscure the results. Thus a negative result in ability to rescue may not be a true negative because the protein is not being expressed at endogenous levels.
4) The assay to determine rescue would be counting spindle orientation through PH3 staining. While these are tumorigenic tissue, capturing actively dividing cells at specific stages in the mitotic process is still a relatively rare event. This experiment alone would take at least 9 months of time.

5) Which Par3 isoform was used for overexpression? Par3 comes in different isoforms due to splicing and potential usage of alternative start codons, the main ones in the epidermis being 180kD, 150 kD and 100 kD, and not all of these isoforms can interact with aPKC, which is known to cooperate with Par3 in driving Ras dependent papilloma formation.

We used the PAR3 isoform that is 180 KDA.

6) The authors refer to symmetric and asymmetric cell division but although not well acknowledged in the skin field, these definitions are coupled to fate. What the authors assess is spindle orientation, an important process but as the authors do not follow the fate of cells with

random spindle orientation there is no direct outcome of fate. Excessive planar spindle orientation which is thought to drive self- renewal to increase the stem cell-like pool of cells has been linked to cancer. In fact, based on the hyperthickening data, in their model spindle orientation seems not to be connected to cell fate but rather random spindles is linked to a disrupted tissue organization observed in this cancer model. For these reasons, it is better to avoid using the terms SCD and ACD which are always connected to cell fate and instead use planar, random or perpendicular spindle orientation as that is what is really assessed by the authors.

We have now corrected this in the text.

7) It was a bit surprising not to see any discussion or reference to the mouse skin cancer models on loss of Par3 and its dual role in keratoacanthoma and papilloma formation as these papers have explored Par3/aPKC expression in SCC (not altered), explored connections between Par3 and Erk, and provided evidence that loss of Par3 and aPKC promotes invasion. Similarly, the Williams group recently showed that loss of AJs controls spindle orientation (Lough et al, Elife 2019) but again this paper is not cited.

We apologize for this and have now included a discussion of this in the manuscript.

8) The authors refer to cell adhesion changes downstream of Par3 and Erk, however, changes in cell-cell adhesion have not been directly addressed. The reduced E-cadherin localization at junctions is suggestive of a loss of adherens junctions, but this loss coiuld be compensated by other adhesion molecues, also as no obvious acantholysis or micro cel-cell blisters are observed. This should be rephrased in the abstract and in other places.

This has been rephrased in the manuscript to ECAD or CLDN1 mediated cell junctions.

9) Figure3C: is there excessive differentiation upon Par3 overexpression?

We tested whether PAR3 overexpression causes excessive differentiation by quantifying epidermal differentiation marker cytokeratin 2 (*KRT2*) expression by qRT-PCR. We found that PAR3/CDK4/RAS xenografts in comparison to control LACZ/CDK4/RAS grafts do not exhibit increased *KRT2* mRNA after vehicle or tamoxifen treatment for 20 days. Please see Supplementary Figure 2B.

10) Spindle orientation measurements: How many spindles were quantified in the different spindle orientation. For Figure 3F (now 3C), at least 3 animals should be used, however, the authors state 2-5 animals. For which groups were only 2 animals used?

The number of spindles quantified for spindle orientation (depicted as radio histograms in Fig.3B) is indicated in the figure as n= #. Thus the total number of spindles quantified per group and timepoint ranged from 58 to 165. We have rephrased the Figure 3B legend to make this clearer. We agree with Reviewer 3 that at least 3 animals for each time point should have been quantified. We have now increased the number of animals quantified to match this criterion. Thus every timepoint and group has at least a minimum of 3 animals. Please see Figure 3B and 3C.

11) How does tamoxifen affect control tissues that do not express the Ras ocongene? As tamoxifen can affect in mice hair cycle, stem cell activation and proliferation and tissue organization, this is an important control. Vehicle control is not tamoxifen treated CDk4 only 3dOrganotypic transplanted, correct?

We agree that tamoxifen may have potential effects on cell proliferation and tissue organization. To determine if tamoxifen itself is causing these effects in non-oncogenic tissue, we treated mice grafted with normal human epidermis (generated from keratinocytes with no exogenous RAS or CDK4) with tamoxifen. After 20 days of tamoxifen treatment, we found the xenografts to be non-hypertrophic and non-hyperproliferative (Supplementary Figure 1G-H). The percent of Ki67 positive cells in the basal layer or the thickness of the normal human epidermis treated with tamoxifen for 20 days was not different from RAS/CDK4 regenerated epidermis treated with VEH

for 20 days (VEH=corn oil injections without tamoxifen) (Supplementary Figure 1G-H). Furthermore, ECAD and PAR3 in normal human epidermis treated with tamoxifen for 20 days were expressed (as observed by IF) with similar localization as CDK4/RAS xenografts treated with VEH. Please see Supplementary Figure 1I-J versus Figure 1D-E.

12) For the first figure it would be good to insert a high magnification.

Unfortunately we don't have access to a higher magnification microscope at our facility.

13) Figure 5: Is upon U01256 Par3 restored on IF? Image not so clear, is it mainly restored in the basal layer?

To better quantify PAR3 expression in TMX20+VEH and TMX20+U0126 treated xenografts, we measured PAR3 protein levels by Western blotting and densitometry. We found U0126 treatment to significantly restore PAR3 protein expression in comparison to VEH treated grafts (Figure 5F). There are higher levels of PAR3 in the basal layer of the epidermis with U0126 treatment. However there is also staining of PAR3 throughout the epidermis as well.

14) Figure 4 last panel Col7 can not be observed in the basement membrane in the controls.

The Col7 can now be seen in the last panel.

15) Figure 2A: DAPI exposure seems different between controls and TMX treated conditions.

This has now been corrected in the new Figure 2A.

Second decision letter

MS ID#: JOCES/2020/249102

MS TITLE: RAS suppression of PAR3 and its effects on SCC initiation and tissue architecture occurs independently of hyperplasia

AUTHORS: Ji Ling, Maria Sckaff, Manisha Tiwari, Yifang Chen, Jingting Li, Jackson Jones, and George Sen
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

The authors present a novel human SCC model system where they test the requirement of PAR3 during RAS-
dependent tumorigenesis.

Comments for the author

Ling et al. present a novel human SCC model system where they show PAR3 operates downstream of RAS-
mediated tumorigenesis. This model will be of great interest to the field and the findings not only confirm existing roles of PAR3, but show how PAR3 regulates SCC tumorigenesis. The revisions have satisfied my critiques and I recommend publication.

Reviewer 2

Advance summary and potential significance to field

These data clearly establish a functional role for PAR3 in the early stages of of Ras driven cutaneous neoplasia, which models human skin SCC. This work will be of interest to a wide range of epithelial biologists. With minor revision, this work would seem appropriate for JCS.

Comments for the author

Authors have adequately addressed the reviewer concerns and the manuscript is significantly strengthened.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed all of my major concerns and this paper is very much improved and now provides a very nice and important contribution to the field.

Comments for the author

The authors have addressed all of my major concerns and this paper is very much improved and now provides a very nice and important contribution to the field.