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Phosphorylation of Pkp1 by RIPK4 regulates epidermal differentiation and skin tumorigenesis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 October 2016

Thank you for the submission of your manuscript (EMBOJ-2016-95679) to The EMBO Journal. Your study has been sent to four referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 states that mechanistic aspects of the study related to tumorigenesis and the signaling downstream of Pkp1 lack conclusiveness and s/he asks you to provide more detailed insights (ref#2, pts. 2,4,5,8). In addition, this referee states discrepancy with previous findings and requests you to improve the discussion of relevant literature (ref#2, pts. 3,6). Further, referee #4 has reservations related to the proteomics data documentation and statistical analysis, which in his/her view weakens the robustness of the study. These issues, together with a number of additional technical requests and controls, raised by the other referees, need to be carefully addressed and clearly responded to in the point-by-point response. Please also see our author guidelines for data representation and statistical analyses.

I judge the comments of the referees to be generally reasonable and we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

Please note that while exploring the relevance of the proposed concept for human skin

tumorigenesis (ref #3; pnt.2) is on itself a valuable aspect, it would in my view not be critically needed for the current study.

REFEREE REPORTS

Referee #1:

In the paper entitles "Phosphorylation of desmosome protein Pkp1 by RIPK4 regulates differentiation of epidermal progenitor cells" the authors present the novel finding that Pkp1 is a substrate for the kinase RIPK4, and phosphorylation of Pkp1 by this kinase is critical for epidermal differentiation. They also present evidence that Ripk4 is a tumor suppressor in a mouse model of skin carcinogenesis. In general the findings of this paper are novel, and the data presented and associated conclusions are sound. However, there are a couple of major criticisms and more minor ones, which the authors should attempt to address if possible.

Main criticisms:

1. The main downside to the study is that the authors are not able to use mouse genetics to full address their hypotheses. Instead of making KO and knock-in mice (e.g. Pkp1 KO, Pkp1-SA, Pkp1-SA), which is obviously time consuming and costly, the authors employ an ex-vitro/engraftment technique to look at the consequence of gene alterations on skin differentiation.

A lot of the results of this paper therefore are based on the technique first discussed in Figure 2D. It would therefore be good to be given more details on how this works. What % of the stratified cell layer (the one exposed to ALI) in vitro looks like stratified skin? Is it homogeneous? How does engraftment look on the actual animals? i.e. does it "take" evenly or only in patches? How do the authors select sections to look at?
- Essential to have images of the various stages of the process - including H&E staining of both the in vitro skin and after the skin graft. This will help identify the skin layers which are hard to see by IF
- How do the authors conduct the experiments shown? How many mice are transplanted? How many sections are taken and from where? How many are quantified and from how many mice? Must all be included in Materials and Methods.

2. Relating to the Ripk4 cKO x K14.Cre mice - how was the Cre inherited in this study? It is essential that the Cre be paternally inherited otherwise deletion occurs in all tissues. There is evidence that the human KRT14 promoter is transcriptionally active in murine oocytes and that the enzyme remains active until after fertilization. (Hafner et al., Genesis, 2004). If this has been done then it should be stated in the materials and methods section.
In addition, as a minimum, the authors needs to show a blot from the epidermis showing that Ripk4 has been deleted in the skin, but not in any other organs (when crossed with the K14-Cre).

3. Figure 6D claims that Pkp1 is the critical substrate of Ripk4, as phosphomimetic completely rescues Ripk4 KO phenotype. To help this data be more convincing, some critical controls should be included:
- Need to put back WT Pkp1 and show it doesn't rescue
- Need to put back Pkp1 S>A and show it doesn't rescue (ideally)
- Would be best to show WT image too (not just Ripk4 KO) and also histology (H&E) if possible of all images

Other comments

Figure 2E -How was this measured? Were multiple images taken? How many mice/engraftments were done per experiment? Please include this information in the M&M section. In addition, could the authors comment on why is there such variability? Seems like most of the thicknesses measured are the same as WT but there are outliers that are thicker. Are the differences between groups statistically significant? Clearly WT and KO do not have the same distribution, i.e. do not have equal variance - so you can't use a T-test.

Figure 2F - "Genomic meta-analysis reveals significant correlation of Pkp1 expression level with differentiation status of mouse skin SCCs. Level of Pkp1 (mRNA) is decreased in poorly differentiated tumor but maintained in SCCs with strong epidermal differentiation or normal epidermal cells (Fig. 2F)."
Would be nice if the authors clarified further in the text or figure legend how this experiment was done:
- What are the SCC cells used/sorted?
- What are Tgfbr2/Ptk2 labels seen in the graph? These are not even mentioned in text or figure legend
- Fold change over what? What is the baseline? Can be indicated on graph or in figure legend.

Figure 2H - Might help to label the bottom 2 panels with some dotted lines to help the reader understand what structures we are looking at

Figure 3B and 3C - Could the authors include some p-values? This seems important given how big the variability is.

Figure 3G - what are the 2 bands of Pkp1? Is it phosphorylation on other sites by other kinase(s)? Might be nice to mention this in the text for clarity.

Figure 4A - This is an important figure and the WB is ugly. It looks likes there actually is some Ripk4 still there in the cKO, but it could also just be dirt/background on the blot, it is hard to tell. Ripk4 is hard to detect by WB so the authors probably had to load a lot of protein to see a signal, but still, is there no way of making it look better? As mentioned in the comments above - authors should show WB from tissue(s) other than skin to show deletion has not occurred.
In addition, Ripk4 in the dermis (if there is any?) will not be deleted in the K14-Cre mice, so perhaps the authors could harvest only the epidermis for WB.

Methods Section - the authors don't mention where the Ripk4 antibody came from.

Figure 4C - Histology H&E image would be a nice complement as it helps get an overview of the skin.

Figure 4E - Again, how was this counted? What does one "dot" represent? One mouse or one image or one section? How many mice were used?

Figure 5D - Would be nice to include statistics

5F and G - In normal skin Ripk4 KO increases expression of Krt10 and Loricrin - why is this different in tumors? Could the authors comment?

5I -See comment for Figure 2F. In addition, would the authors want to include a regression line and R2 value?

Figure 7C and 7D - This doesn't look very convincing, especially the western blot. The authors claim that level of phospho-Erk is significantly elevated but then they do not show any statistics, this should be included or "statistically significant" taken out

Figure 7E - As a control the authors should show the IP: Pkp1 and IB:Pkp1 blot - otherwise how does the reader know the IP has worked?

Figure 7F - Data provides some evidence for the hypothesis the authors are putting forward but by no means conclusively demonstrates it. For example - what evidence is there that binding of Pkp1 to SHOC2 disrupts the Ras/Raf complex? Would really add weight to the finding if the authors could find a way of addressing this.

Discussion

The Discussion is just another summary of the findings of the paper plus a bit more background

information from the literature. This section could be made a lot for interesting and thought provoking, by discussing a few key issues.
For example, what do the authors predict the Pkp1 KO mouse phenotype to be? Do the authors think that Pkp1 is the sole critical substrate of Ripk4 in epidermal development? The Pkp1-SE rescue data (Figure 6E) suggests that this is the case, however Irf6 has also been reported to be important. If Pkp1 is the sole critical substrate then it should completely encompass the Ripk4 KO phenotype at the very least.
The issue of why Ripk4 is over-expressed in certain tumor types, but also act as a tumor suppressor is puzzling. Why could this be? Is the Ripk4-Pkp1 signaling axis studied in this paper present in other tumor types other than SCCs (presumably not)?
Lastly, why are genes that are mutated in SCCs frequently involved in epidermal differentiation e.g. Irf6, Notch, p63 etc? Is it just because removing these genes increases the proliferative potential of the cells of the skin (makes them more "stem-like") or is it some other mechanism (e.g. response to damage)?

Typos

When authors say Supplementary Figure 5A - do they mean 6A?

Referee #2:

In their manuscript "Phosphorylation of Desmosome Protein Pkp1 by RIPK4 Regulates Differentiation of Epidermal Progenitor Cells and Skin Tumorigenesis" the authors cover several major conceptual and technical areas of inquiry. First, they utilize quantitative proteomics techniques to assess post-translational modifications of proteins in mouse epidermal progenitor cells undergoing differentiation induced by calcium. They uncover a number of protein phosphorylation differences in undifferentiated vs. early differentiating cell populations, then employ data analysis methods to functionally cluster the types of proteins that were most frequently modified, finding the majority to be cell membrane, junction, and adhesion molecules (Figure 1). They use this analysis as a springboard to narrow their focus to plakophilin 1 (Pkp), using as a premise their identification of three post-translational modifications identified on this desmosomal armadillo protein. Before examining the role of these modifications, they characterize the role of Pkp1 in differentiation and tumor progression (using CRISPR-Cas9 to remove Pkp from mouse progenitors followed by analysis in vitro and in engrafted mice). The authors next move to mechanistic studies of Pkp1 phosphorylation, carrying out a kinome screen and identifying RIPK4 as responsible for Pkp1 phosphorylation. They go on to generate and characterize a RIPK4 KO, and finally, explore functional connections among these players, focusing on a previously identified signaling hub involving Desmoglein 1, Erbin, and SHOC2 and the Erk/MAPK pathway.

The authors conclude that PKP1 and RIPK4 deficiency result in similar epidermal differentiation defects, and suggest that impaired differentiation leads to increased skin tumorigenesis. They confirm that PKP1 phosphorylation is reduced in the RIPK KO mice, and, importantly, show that the Pkp1 SE mutant phosphomimetic is able to rescue Krt14 and Krt10 staining (comparing grafts from RIPK4 KO cells with RIPK4 KO cells plus Pkp1 SE). In the final figure the authors report an interaction between WT Pkp1 and the phosphomimetic form with SHOC2, drawing the conclusion that Pkp1-SHOC2 binding can enhance epidermal differentiation by blocking the Ras/MAPK signaling pathway.

Overall this is a provocative, ambitious study, with broad ranging questions, approaches, outcomes and conclusions. While there are strong mechanistic components, because of the ambitious nature of the study many aspects are superficially addressed. Some of the mechanistic aspects covered (e.g. those in Figure 7), would need to be more complete to be appropriate for publication in EMBO J. Overall, while there is a lot to interest the EMBO J. readership in this manuscript, it is not suitable for publication in its present form. It would take some major streamlining. I envision a recrafted manuscript with some gaps filled in (possibly removing tumor studies but expanding on role of Pkp1 and its PTMs in differentiation) would make a strong contribution.

Specific comments:

1) The premise for narrowing the focus so abruptly to Pkp1 is not particularly well-articulated. Indeed, several other armadillo proteins were also modified, which could also have functional importance in adhesion, signaling, and/or differentiation. The reader is left wondering about the respective roles of other modified proteins.

2) The conclusion is drawn that Pkp1 is important for epidermal differentiation based on a limited analysis of K10/K14 in KO grafts. No explanation is given for the epidermal thickening. There is lost opportunity to assess potentially mechanistically revealing features, such as cell junction protein expression/localization and cytoarchitecture/trans-epidermal water loss/mechanical strength. Probably most relevant to later aspects of the study would be desmoglein 1, which was previously shown to scaffold a complex regulating Shoc2 (a focus of the last figure).

3) The authors fail to mention or compare their results with recent results from a Pkp1 knockout mouse (Ritscher, et al. 2016. Growth Retardation, loss of desmosomal adhesion, and impaired tight junction function identify a unique role of plakophilin 1 in vivo. JID 136: 1471.)

4) While the concept that aberrant "epidermal differentiation contributes heavily to skin tumorigenesis" is a reasonable one, the rationale to include these studies here is not well-established. The conclusion that enhanced tumorigenesis and progression is due to aberrant epidermal differentiation is based on limited analysis of Krt10 and loricrin. No proliferation or apoptosis assays were performed and no further characterization of differences between WT and Pkp1 KO tumors in the ha-Ras genetic background were included. Overall the analysis of tumorigenesis is superficial.

5) Data supporting the importance of Pkp1 head domain phosphorylation for epidermal structure and differentiation as determined by K14 and K10 staining are among the strongest in the manuscript (SA vs. SE mutant data). More careful characterization of desmosomal component structure and localization as well as keratin organization would strengthen these results. In particular, is desmoglein 1 localization at the membrane impacted by Pkp1 head domain phosphorylation? The answer to this question may provide clues as to how Pkp1 impacts epidermal differentiation since Dsg1 itself controls epidermal differentiation and signaling. Data (immunoprecipitation) in Figure 7 seem to indicate that Pkp1 and Dsg1 are not in a complex in this study. However, Dsg1 is very insoluble and it isn't clear whether this contributes to the failure to observe it in a complex with Pkp1. The only other desmosomal component analyzed in this study is Dsp in Figure 7 and the images are not high enough resolution to draw the conclusion that loss of Pkp1 does not significantly alter the desmosome structure. Further, conclusions are drawn about localization of Pkp1 itself (being present in the cytoplasm and at the membranes, but not in the nucleus). However, the staining was done after fixation in 4% paraformaldehyde and different fixation methods may be required to observe nuclear localization of Pkp1 as previously reported.

6) A recent paper (Kwa et al Journal of Biological Chemistry Vol 289 No 45 pp 31077-31087 November 7, 2014) showed involvement of RIPK4 in controlling expression of keratinocyte differentiation regulators. The current study knocks out RIPK4 in mouse skin (an important advance towards elucidating how RIPK4 regulates epidermal differentiation). However, analysis of differentiation and desmosome components (esp. Dsg1) is given short shrift. The 2014 Kwa et al. paper should be referenced, as well (a 2015 paper by the same lead author was referenced, but both are important to the current study).

7) Plakoglobin and Desmoplakin also exhibited changes in phosphorylation in the RIPK4 KO samples, an important finding that could have implications for differentiation, signaling, and cytoskeletal architecture.

8) In the final figure the authors address the signaling pathway by which Pkp1 phosphorylation impacts epidermal differentiation, focusing on a previously reported signaling hub involving Desmoglein 1, Erbin, and SHOC2. They find an interaction between both WT Pkp1 and the phosphomimetic form of Pkp1 with SHOC2, drawing the conclusion that Pkp1-SHOC2 binding can enhance epidermal differentiation by interfering with the Ras/MAPK signaling pathway. These data are interesting but not fully fleshed out. In this regard, see comment regarding Dsg1 in point 5 above. In addition, they do not show whether Pkp1 can bind SHOC 2 or not in RIPK4 KO cells, even though they state in the discussion section that Pkp1 can associate with SHOC2 after RIPK4-

mediated phosphorylation. Overall the mechanism proposed in Figure 7 could be strengthened.

Additional comments to authors:

1) Many of the references utilized in the manuscript are dated, particularly the references associated with the introduction of epidermal differentiation and cell adhesion molecule functions.
2) Language editing by a native speaker or use of a professional editing service would help to improve the text.
3) The Pkp1-deficient cells grafted onto mice in the first portion of the study should be consistently referred to as Pkp1-KO rather than calling them Pkp1 mutant (It is my understanding that CRISPR-cas9 deleted the gene, but did not cause mutations and this could increase confusion since Pkp1 mutations in phosphorylation sites are studied later in the manuscript).
4) Figure 3b and 3c - should the figure labels read Pkp1KO+Pkp1SE rather than SD?
5) In the text for Figure 4 results, it is stated that Krt-5 positive cells extend to the suprabasal layers, while in the figure it says Krt-14. Which is correct?
6) Is there an explanation for why there is not a difference (authors say "comparable") in Ha-ras induced pErk levels in WT and Pkp1KO cells (supplementary Fig 2) while Pkp1 KO alone leads to increased pErk, the subject of Figure 7? To me it actually seems as if the KO levels may be higher in Supp. Fig. 2.
7) The discussion section is quite broad and some parts seem more suited for an introduction or review rather than a discussion.

Referee #3:

The authors used a well-established technology, the SILAC mouse technology coupled to high-resolution mass spectrometry, to quantify phosphoproteomic changes in keratinocyte in culture according to their state of differentiation. The goal of the study was to study how changes in the phosphoproteome regulate self-renewal and differentiation of epidermal stem and progenitor cells. Not surprisingly, the authors found that desmosomal proteins represent an enriched-group of phosphorylated proteins during differentiation. The manuscript focuses on one of them, Pkp1. Using carefully controlled experiments the authors identified the phosphorylation site in Pkp1 that is crucial for skin epidermal differentiation and the kinase responsible for the phosphorylation. They further used mouse genetic to show functionally the role of this kinase in skin differentiation and carcinogenesis. Study of Pkp1 and RIPK4 regulating differentiation is strong but the mechanism linked to tumorigenesis is less clear especially regarding to the role of SHOC2-Ras/Raf-Erk axis. Overall, this is a well-written study that tackles important questions about what control epidermal differentiation and skin tumorigenesis at the molecular level.

Specific major concerns essential to be addressed to support the conclusions

1) Figure 2F: This figure is very confusing and the figure legend does not explain clearly where the poorly differentiated tumor comes from. Moreover, the rational to use a6+b1+ cells, the meaning of Tgfbr2 or Ptk2 in the figure are unclear. Along these lines, the authors should discuss the work of Zanivan et al., 2013 who identify using the SILAC technology desmosomal proteins Dsg1 and Dsg2 significantly downregulated during skin differentiation and SCC progression.

2) Human skin tumors are easy to obtain and authors should look if Pkp1 and Ripk4 are found downregulated according to the stage of differentiation.

3) The authors should clarify in supplemental figure 3 the size differences between the WT and the exogenous Pkp1 expression and if the doublet bands correspond to proteolysis.

4) Figure 4: It will be useful to show where RIPK4 is expressed in the epidermis and confirm its loss of expression in the cKO backskin. Also it is expected that proliferation should not change in the cKO skin but this need to be shown.

5) The quality of the histological staining to characterize the RIPK4 cKO SCC is poor and the analysis should be confirmed by a pathologist. The authors wrote an entire paragraph (p10, line 6) comparing the WT and RIPK4 cKO tumors but the images shown in supplemental figure 5 do not

support the description. The images shown do not reflect hematoxylin and eosin staining which is usually blue, red and pink and not brown as depicted here. Hematoxylin and Eosin staining on paraffin-embedded tissues is commonly used to preserve the tissue architecture. This comment is also valid for the histology in supplemental figure 2E.

6) Figure 5H: it is not clear on the rationale to look for expression level of RIPK4 and PKP1 in hair follicle stem cells and on the various mouse models used, similarly to figure 2F. Degree of differentiation in SCC could be analyzed on the TPA, papilloma and SCC (DMBA) models.

Minor concerns that should be addressed

1) Please reference or show the data showing that 12h of calcium switch is a good time point to see early differentiation as not all readers are skin experts.

2) Figure 2D: it will be useful to show where Pkp1 is expressed in the epidermis here (and not in the Figure 7) and confirm its loss of expression in the KO skin graft.

3) Supplemental figure 2E: should be properly labeled as WT+V12Hras and Pkp1-KO+ V12Hras.

4) Western blots are all lacking size markers and many of them seems overloaded (Figure 4A, Supplemental Figure 2). The quality of the western blots could be improved.

5) In Figure 4 the authors described in the text the use of Krt-5 and labeled the figure with Krt-14.

6) p11 line 3: the authors referenced supplemental figure 5A, 5B instead of 6A, 6B.

7) Figure 7: age of the tissue should be mentioned.

Referee #4:

Lee et al. submitted a manuscript on phosphorylation of Pkp1 by RIPK4 and its role in regulation of epidermal differentiation and skin tumorigenesis. The authors performed several SILAC-based phosphoproteome analyses of differentiated and undifferentiated keratinocytes. In their dataset, several phosphorylation sites on the Pkp1 appeared to be upregulated, which motivated the authors to perform follow-up experiments linking Pkp1 phosphorylation with RIPK4 activity, epidermal differentiation and skin tumorigenesis. As requested by the Editor, and in the interest of time, I here focus solely on the proteomics part(s) of the study. Proteomics was done using state-of-the-art methodology (SILAC), equipment (Q-Exactive) and software (MaxQuant suite from the Mann lab). However, key information on the experiments is either unclear or missing. This includes:

Major points:

• Description of the statistical treatment of significant (phospho)peptide ratios - the authors used a cutoff of 2-fold change, which is completely arbitrary; this is concerning, given the fact that the median of measured SILAC ratios is not at log2=0 (it is around log2=-0.5, see figure 1B). This means that negative ratios will be enriched in "significantly" changing proteins. The authors should rather use the "significance B" value provided by the software.

• The shift towards negative ratios (see above) may be caused by technical factors, such as incomplete labeling or a large mixing error; this should be discussed in the text (and the labeling/mixing data should be submitted as supplementary material)

• Phosphopeptide ratios should be normalized with (unmodified) protein ratios to ensure that the observed change in protein phosphorylation is not driven by gene expression (rather than kinase activity)

• Supplementary tables should contain information on localization probability (and significance B) and only localized sites (e.g. probability >75%) should be discussed

• The authors should provide annotated spectra of (at least) the three Pkp1 phosphorylation sites, demonstrating their correct localization on the peptide backbone

Minor points

• Brief description of the phosphopeptide enrichment protocol (or a reference) is missing

• Text should be thoroughly edited for proper use of English grammar

It is possible that the authors observed most of these criteria, but this is not visible from the text. Until these points are fully addressed, the interpretation of proteomics results may be misleading and does not meet the standards of the EMBO J.

1st Revision - authors' response 08 February 2017

We are delighted that the four reviewers found our work to be novel and of high interest to the readership of *EMBO J*. Each reviewer has made enormously helpful comments. We've now fully addressed these issues, and in doing so, have substantially improved the manuscript and its impact. We've conducted the various experiments suggested by each reviewer and revised the manuscript accordingly as we delineate below. We really thank all the reviewers for all of their constructive comments!

Reviewer #1 (Remarks to the Author):

In the paper entitles "Phosphorylation of desmosome protein Pkp1 by RIPK4 regulates differentiation of epidermal progenitor cells" the authors present the novel finding that Pkp1 is a substrate for the kinase RIPK4, and phosphorylation of Pkp1 by this kinase is critical for epidermal differentiation. They also present evidence that Ripk4 is a tumor suppressor in a mouse model of skin carcinogenesis. In general the findings of this paper are novel, and the data presented and associated conclusions are sound. However, there are a couple of major criticisms and more minor ones, which the authors should attempt to address if possible.

We thank the reviewer for appreciating the novelty of our work.

Main criticisms:

1. The main downside to the study is that the authors are not able to use mouse genetics to full address their hypotheses. Instead of making KO and knock-in mice (e.g. Pkp1 KO, Pkp1-SA, Pkp1-SA), which is obviously time consuming and costly, the authors employ an ex-vitro/engraftment technique to look at the consequence of gene alterations on skin differentiation.

A lot of the results of this paper therefore are based on the technique first discussed in Figure 2D. It would therefore be good to be given more details on how this works. What % of the stratified cell layer (the one exposed to ALI) in vitro looks like stratified skin? Is it homogeneous? How does engraftment look on the actual animals. i.e. does it "take" evenly or only in patches? How do the authors select sections to look at?
- Essential to have images of the various stages of the process - including H&E staining of both the in vitro skin and after the skin graft. This will help identify the skin layers, which are hard to see by IF
- How do the authors conduct the experiments shown? How many mice are transplanted? How many sections are taken and from where? How many are quantified and from how many mice? Must all be included in Materials and Methods.

We thank the reviewer for this suggestion. As mentioned by the reviewer, the traditional mouse genetics with KO or transgenic are time-consuming and expensive. To circumvent this technical barrier, we have developed a skin transplantation system that can efficiently test skin stratification *in vivo*. The establishment of this novel platform has been described in our published works (Liu et al, 2015; Yue et al, 2016). The skin grafts were developed from organotypic culture of primary mouse keratinocytes, and can be well taken when grafted to nude mice. Regenerated skin from WT

keratinocytes exhibits normal skin histology and stratification. We usually use *H2B-RFP* as a marker for grafted skin (Liu et al, 2015; Yue et al, 2016).
As suggested, we included H&E staining of skin before and after grafting in Supplementary Fig. 2B and C. We have also revised the Material & Methods section to include all the details for skin grafting experiments.

2. Relating to the Ripk4 cKO x K14.Cre mice - how was the Cre inherited in this study? It is essential that the Cre be paternally inherited otherwise deletion occurs in all tissues. There is evidence that the human KRT14 promoter is transcriptionally active in murine oocytes and that the enzyme remains active until after fertilization. (Hafner et al., Genesis, 2004). If this has been done then it should be stated in the materials and methods section.
In addition, as a minimum, the authors needs to show a blot from the epidermis showing that Ripk4 has been deleted in the skin, but not in any other organs (when crossed with the K14-Cre).

We thank the reviewer for pointing this out. To avoid potential maternal effect, we generally use male mice with *K14-Cre* transgene during breeding. Our *K14-Cre* strain was developed by Dr. Elaine Fuchs's lab, and has been used widely for skin conditional knockout (Vasioukhin et al, 1999). It is very unlikely that the *Cre* will delete *RIPK4* in other tissues, as total KO of *RIPK4* is perinatally lethal (Holland et al, 2002). To confirm the specificity, we have included western blotting results to show RIPK4 is present in dermis and liver in the cKO mice (Supplementary Fig. 4C).

3. Figure 6D claims that Pkp1 is the critical substrate of Ripk4, as phosphomimetic completely rescues Ripk4 KO phenotype. To help this data be more convincing, some critical controls should be included:
- Need to put back WT Pkp1 and show it doesn't rescue
- Need to put back Pkp1 S>A and show it doesn't rescue (ideally)
- Would be best to show WT image too (not just Ripk4 KO) and also histology (H&E) if possible of all images

We thank the reviewer for the suggestion. We have performed staining and quantifications as suggested. The results are included in the revised manuscript (Fig. 6D and supplementary Fig. 6C-F). The results strongly support our hypothesis that RIPK4-mediated phosphorylation of Pkp1 promotes skin epidermal differentiation.

Other comments

Figure 2E -How was this measured? Were multiple images taken? How many mice/engraftments were done per experiment? Please include this information in the M&M section. In addition, could the authors comment on why is there such variability? Seems like most of the thicknesses measured are the same as WT but there are outliers that are thicker. Are the differences between groups statistically significant? Clearly WT and KO do not have the same distribution, i.e. do not have equal variance - so you can't use a T-test.

We have revised the M&M sections as suggested to include all the experimental details (please also see our response to major criticism point 1). All the experiments had more than 3 biological replicates (independent skin grafts). For phenotypic analysis, at least 3 sections were taken from each graft for analysis and quantification by staining. Each dot (data point) in the graft represents average thickness from one field of the section.

Variation indeed exists for *in vivo* samples, even for the WT tissue. The difference between WT and KO tissue is statistically significant ($P<0.05$, Mann-Whitney U test).

Figure 2F - "Genomic meta-analysis reveals significant correlation of Pkp1 expression level with differentiation status of mouse skin SCCs. Level of Pkp1 (mRNA) is decreased in poorly differentiated tumor but maintained in SCCs with strong epidermal differentiation or normal epidermal cells (Fig. 2F)."
Would be nice if the authors clarified further in the text or figure legend how this experiment was done:

- What are the SCC cells used/sorted?
- What are Tgfbr2/Ptk2 labels seen in the graph? These are not even mentioned in text or figure legend
- Fold change over what? What is the baseline? Can be indicated on graph or in figure legend.

Revised the text as suggested. "To test for a role of Pkp1 in skin carcinogenesis, we first explored its relative expression levels on published microarray data sets that compared transcriptomes of tumor propagating cancer cells (TPCs) isolated from moderately to poorly differentiated control and *Tgfbr2*^{KO} SCC and well differentiated *Ptk2*^{KO} and *Tgfbr2*^{KO}/*Ptk2*^{KO} SCCs to normal skin epithelial stem and progenitor cells (Schober & Fuchs, 2011). *Pkp1* (mRNA) is consistently reduced in TPCs compared to normal skin epithelial stem and progenitor cells with lowest expression levels in highly tumorigenic *Tgfbr2*^{KO} TPCs (Fig. 2F). These data suggest that reduced *Pkp1* expression might accelerate skin carcinogenesis and progression."
The baseline is the level of *Pkp1* in normal epithelial progenitor cells (indicated in the new Figure legend).

Figure 2H - Might help to label the bottom 2 panels with some dotted lines to help the reader understand what structures we are looking at

The tumors developed from *Pkp1* KO cells are more advanced and invasive. Unlike WT tumors, which still exhibit epidermal differentiation and contain continuous layer of basal-like cells (with beta4-integrin), the KO tumor are very aggressive and disorganized (as shown in Fig. 2H with beta 4 integrin staining). It is difficult to use "dotted lines" to distinguish epithelial tissue in the KO tumors. We have revised the Figure legend to clarify this.

Figure 3B and 3C - Could the authors include some p-values? This seems important given how big the variability is.

We have performed statistical analysis with one-way ANOVA (tukey test for the mean comparisons). For 3B, while WT, Pkp1 KO+ PKP1, and Pkp1 Ko + Pkp1-SE show no significant difference, Pkp1 KO and KO + Pkp SA are significantly thicker for Krt14 layers ($P<0.05$). Similarly, in 3C, expression of WT *Pkp1* or SE mutant of *Pkp1* significantly reduced the thickness of Krt10 layer, whereas SA mutant increases it ($p<0.05$).

Figure 3G - what are the 2 bands of Pkp1? Is it phosphorylation on other sites by other kinase(s)? Might be nice to mention this in the text for clarity.

Exogenously expressed *Pkp1* exhibits two bands in immunoblots. The lower band (star) likely represents degradation product of over-expressed protein. Figure legends were revised accordingly.

Figure 4A - This is an important figure and the WB is ugly. It looks likes there actually is some Ripk4 still there in the cKO, but it could also just be dirt/background on the blot, it is hard to tell. Ripk4 is hard to detect by WB so the authors probably had to load a lot of protein to see a signal, but still, is there no way of making it look better? As mentioned in the comments above - authors should show WB from tissue(s) other than skin to show deletion has not occurred.
In addition, Ripk4 in the dermis (if there is any?) will not be deleted in the K14-Cre mice, so perhaps the authors could harvest only the epidermis for WB.

We have repeated the immunoblot as suggested (Fig. 4A, skin epidermis). We have also examined RIPK4 level in other tissue and organs including dermis as suggested (Supplementary Fig. 4C).

Methods Section - the authors don't mention where the Ripk4 antibody came from.

RIPK4 antibody was obtained from abnova.

Figure 4C - Histology H&E image would be a nice complement as it helps get an overview of the skin.

H/E staining images were included in the revised manuscript (Supplementary Fig. 4D).

Figure 4E - Again, how was this counted? What does one "dot" represent? One mouse or one image or one section? How many mice were used?

We have revised the M&M sections as suggested to include all the experimental details (please also see our response to major criticism point 1). All the experiments had more than 3 biological replicates (independent skin grafts). For phenotypic analysis, at least 3 sections were taken from each graft for analysis and quantification by staining. Each dot (data point) in the graft represents average thickness from one field of the section.

Figure 5D - Would be nice to include statistics

Statistics included as suggested.

5F and G - In normal skin Ripk4 KO increases expression of Krt10 and Loricrin - why is this different in tumors? Could the authors comment?

We have revised the Discussion section (Page 17, last paragraph) to include this issue: "Our results suggest that RIPK4/Pkp1 pathway regulates epidermal differentiation in both normal skin and skin tumors. Deletion of *RIPK4* or *Pkp1* leads to expansion of both basal cell layer and spinous (early differentiation) layer in skin. Granular layer (late differentiation) is not changed significantly in the KO skin. In skin tumors, interestingly, loss of *RIPK4* or *Pkp1* causes significant reduction of expression for both early and late differentiation markers. The difference could be due to the mutations or epigenetic changes in the skin tumor cells. For example, constitutively active Ras or MAP kinase pathway in the tumor cells can suppress keratinocyte differentiation by itself (Dajee et al, 2002; Khavari & Rinn, 2007; Tarutani et al, 2003). Loss of *RIPK4* or *Pkp1* in this background would likely result in more severe defects in epidermal differentiation."

5I -See comment for Figure 2F. In addition, would the authors want to include a regression line and R2 value?

We thank the reviewer for pointing this out. We have revised the text to clarify this issue: "*RIPK4* mutations have been detected in human head and neck SCC, suggesting a critical function of RIPK4 in squamous differentiation and carcinogenesis (Stransky et al, 2011). In addition to mutations, expression of *RIPK4* may be blunted when tumors initiate and progress to malignant cancers. Like Pkp1 (Fig. 2F), *Ripk4* expression is also blunted in TPCs of SCC, compared to normal skin epithelial stem and progenitor cells, where poorly differentiated, aggressively growing tumors express the lowest amount of Ripk4 (Fig. 5H). Direct comparisons between Ripk4 and Pkp1 expression in these TPCs revealed a positive correlation (R^2=0.58, p=0.0171) of these markers, with highest expression in inter-follicular progenitor cells and lower expression in hair follicle stem cells (Fig. 5I). Together, our data in normal skin and tumors suggest that the relative expression and activity of Ripk4 and Pkp1 could be explored as a prognostic measure for tumor differentiation and clinical outcome in future studies."
R^2 and regression line are included in the revised manuscript.

Figure 7C and 7D - This doesn't look very convincing, especially the western blot. The authors claim that level of phospho-Erk is significantly elevated but then they do not show any statistics, this should be included or "statistically significant" taken out

The difference in Phopho-Erk is mild but statistically significant. We have performed statistical analysis as suggested (one-way ANOVA).

Figure 7E - As a control the authors should show the IP: Pkp1 and IB:Pkp1 blot - otherwise how does the reader know the IP has worked?

IP blot included as suggested (Fig. 7D).

Figure 7F - Data provides some evidence for the hypothesis the authors are putting forward but by no means conclusively demonstrates it. For example - what evidence is there that binding of Pkp1 to SHOC2 disrupts the Ras/Raf complex? Would really add weight to the finding if the authors could find a way of addressing this.

Our results suggest that Pkp1 phosphorylation by RIPK4 regulates keratinocyte differentiation through the Ras/Raf adaptor protein Shoc2, because 1) Pkp1 but not Pkp1-SA mutant exhibits strong binding with Shoc2, and 2) WT Pkp1 but not Pkp1 SA mutant can suppress level of Phospho-Erk in keratinocytes. Additionally, deletion of *RIPK4* reduces binding between Shoc2 and Pkp1 (Fig. 7F). Although our current results are all consistent with this hypothesis, we agree with the reviewer that we cannot conclusively prove the hypothesis at this stage, and future work will be essential to delineate the downstream signaling network. We have revised the text and the discussion to address this issue.

Discussion

The Discussion is just another summary of the findings of the paper plus a bit more background information from the literature. This section could be made a lot for interesting and thought provoking, by discussing a few key issues.
For example, what do the authors predict the Pkp1 KO mouse phenotype to be? Do the authors think that Pkp1 is the sole critical substrate of Ripk4 in epidermal development? The Pkp1-SE rescue data (Figure 6E) suggests that this is the case, however Irf6 has also been reported to be important. If Pkp1 is the sole critical substrate then it should completely encompass the Ripk4 KO phenotype at the very least.
The issue of why Ripk4 is over-expressed in certain tumor types, but also act as a tumor suppressor is puzzling. Why could this be? Is the Ripk4-Pkp1 signaling axis studied in this paper present in other tumor types other than SCCs (presumably not)?
Lastly, why are genes that are mutated in SCCs frequently involved in epidermal differentiation e.g. Irf6, Notch, p63 etc? Is it just because removing these genes increases the proliferative potential of the cells of the skin (makes them more "stem-like") or is it some other mechanism (e.g. response to damage)?

We are very grateful to the reviewer for the suggestion. We have revised the Discussion accordingly to include all the points.

Typos: When authors say Supplementary Figure 5A - do they mean 6A?

Corrected.

Referee #2:

In their manuscript "Phosphorylation of Desmosome Protein Pkp1 by RIPK4 Regulates Differentiation of Epidermal Progenitor Cells and Skin Tumorigenesis" the authors cover several major conceptual and technical areas of inquiry. First, they utilize quantitative proteomics techniques to assess post-translational modifications of proteins in mouse epidermal progenitor cells undergoing differentiation induced by calcium. They uncover a number of protein phosphorylation differences in undifferentiated vs. early differentiating cell populations, then employ data analysis methods to functionally cluster the types of proteins that were most frequently modified, finding the majority to be cell membrane, junction, and adhesion molecules (Figure 1). They use this analysis as a springboard to narrow their focus to plakophilin 1 (Pkp), using as a premise their identification of three post-translational modifications identified on this desmosomal armadillo protein. Before examining the role of these modifications, they characterize the role of Pkp1 in differentiation and tumor progression (using CRISPR-Cas9 to remove Pkp from mouse progenitors followed by analysis in vitro and in engrafted mice). The authors next move to mechanistic studies of Pkp1 phosphorylation, carrying out a kinome screen and identifying RIPK4 as responsible for Pkp1 phosphorylation. They go on to generate and characterize a RIPK4 KO, and finally, explore functional connections among these players, focusing on a previously identified signaling hub involving Desmoglein 1, Erbin, and SHOC2 and the Erk/MAPK pathway.

The authors conclude that PKP1 and RIPK4 deficiency result in similar epidermal differentiation defects, and suggest that impaired differentiation leads to increased skin tumorigenesis. They confirm that PKP1 phosphorylation is reduced in the RIPK KO mice, and, importantly, show that the Pkp1 SE mutant phosphomimetic is able to rescue Krt14 and Krt10 staining (comparing grafts from RIPK4 KO cells with RIPK4 KO cells plus Pkp1 SE). In the final figure the authors report an

interaction between WT Pkp1 and the phosphomimetic form with SHOC2, drawing the conclusion that Pkp1-SHOC2 binding can enhance epidermal differentiation by blocking the Ras/MAPK signaling pathway.

Overall this is a provocative, ambitious study, with broad ranging questions, approaches, outcomes and conclusions. While there are strong mechanistic components, because of the ambitious nature of the study many aspects are superficially addressed. Some of the mechanistic aspects covered (e.g. those in Figure 7), would need to be more complete to be appropriate for publication in EMBO J. Overall, while there is a lot to interest the EMBO J. readership in this manuscript, it is not suitable for publication in its present form. It would take some major streamlining. I envision a recrafted manuscript with some gaps filled in (possibly removing tumor studies but expanding on role of Pkp1 and its PTMs in differentiation) would make a strong contribution.

We are very grateful to the reviewer for the constructive suggestions.

Specific comments:

1) The premise for narrowing the focus so abruptly to Pkp1 is not particularly well-articulated. Indeed, several other armadillo proteins were also modified, which could also have functional importance in adhesion, signaling, and/or differentiation. The reader is left wondering about the respective roles of other modified proteins.

We have identified several desmosomal proteins, which display changed phosphorylation during epidermal differentiation. We focused our analysis on Pkp1 in this study because it is one of the most significantly modified proteins with multiple upregulated phosphorylation sites upon calcium shift. Three prominent phosphorylation sites at the head domain of Pkp1 are all significantly upregulated in differentiated cells. In addition, previous studies suggest Pkp1 could be critically involved in epidermal differentiation, both from analysis of EDSF patient skin and recent KO of *Pkp1* in mice.
We agree with the reviewer that modification of other desmosomal and armadillo proteins could play important roles as well. It will be interesting and important to address their modification and potential signaling role in the future.
We have revised the manuscript in both Results and Discussion to address this issue.

2) The conclusion is drawn that Pkp1 is important for epidermal differentiation based on a limited analysis of K10/K14 in KO grafts. No explanation is given for the epidermal thickening. There is lost opportunity to assess potentially mechanistically revealing features, such as cell junction protein expression/localization and cytoarchitecture/trans-epidermal water loss/mechanical strength. Probably most relevant to later aspects of the study would be desmoglein 1, which was previously shown to scaffold a complex regulating Shoc2 (a focus of the last figure).

We thank the reviewer for this suggestion. As suggested, we have tested desmosome and tight junctions in the KO skin by staining of desmoglein 1, desmoplakin 1, and ZO-1 (Fig 7A and Supplementary Fig. 2F, 3B). We have also tested desmoglein 1 localization in different rescued skin grafts. The new results are included in the revised manuscript. Consistent with previous report for human EDSF syndrome and recent publication with mouse Pkp1 KO, keratinocytes with *Pkp1* deletion still exhibit intercellular localization of desmosomal proteins, such as desmoglein 1 and desmoplakin 1, however, we can see more diffusive and intracytoplasmic staining of Dsp1 and DSG1 in the KO skin. Tight junction marker, ZO-1 shows an abnormal pattern in the KO skin. ZO staining is significantly discontinuous and reduced in the KO skin. Aberrant tight junction could lead to barrier defect. However, because of the size and physical features of the skin grafts, we cannot directly test it in our model. Inside-out barrier defect and mechanical strength studies have already been carried out in the recent mouse KO study (Rietscher et al, 2016) (included in the discussion). In the revised manuscript, we have also provided more discussion of the *Pkp1* KO phenotype, including epidermal thickening, expansion of Krt14 and Krt10.

3) The authors fail to mention or compare their results with recent results from a Pkp1 knockout mouse (Ritscher, et al. 2016. Growth Retardation, loss of desmosomal adhesion, and impaired tight junction function identify a unique role of plakophilin 1 in vivo. JID 136: 1471.)

We thank the reviewer for pointing this out. It has been included in the revised Results and Discussion sections.

4) While the concept that aberrant "epidermal differentiation contributes heavily to skin tumorigenesis" is a reasonable one, the rationale to include these studies here is not well-established. The conclusion that enhanced tumorigenesis and progression is due to aberrant epidermal differentiation is based on limited analysis of Krt10 and loricrin. No proliferation or apoptosis assays were performed and no further characterization of differences between WT and Pkp1 KO tumors in the ha-Ras genetic background were included. Overall the analysis of tumorigenesis is superficial.

We analyzed cell proliferation and apoptosis in the WT and *Pkp1* KO tumors as suggested. Deletion of *Pkp1* in Ras-induced skin tumor leads to increased apoptosis and slightly reduced cell proliferation (both are statistically significant, student t-test) (Supplementary Fig. 2I-J). Together, the data strongly suggest that enhanced tumorigenesis of Pkp1 KO cells is most likely due to suppressed cell differentiation.

5) Data supporting the importance of Pkp1 head domain phosphorylation for epidermal structure and differentiation as determined by K14 and K10 staining are among the strongest in the manuscript (SA vs. SE mutant data). More careful characterization of desmosomal component structure and localization as well as keratin organization would strengthen these results. In particular, is desmoglein 1 localization at the membrane impacted by Pkp1 head domain phosphorylation? The answer to this question may provide clues as to how Pkp1 impacts epidermal differentiation since Dsg1 itself controls epidermal differentiation and signaling. Data (immunoprecipitation) in Figure 7 seem to indicate that Pkp1 and Dsg1 are not in a complex in this study. However, Dsg1 is very insoluble and it isn't clear whether this contributes to the failure to observe it in a complex with Pkp1. The only other desmosomal component analyzed in this study is Dsp in Figure 7 and the images are not high enough resolution to draw the conclusion that loss of Pkp1 does not significantly alter the desmosome structure. Further, conclusions are drawn about localization of Pkp1 itself (being present in the cytoplasm and at the membranes, but not in the nucleus). However, the staining was done after fixation in 4% paraformaldehyde and different fixation methods may be required to observe nuclear localization of Pkp1 as previously reported.

We analyzed DSG1 localization in skin generated from WT, Pkp1 KO, KO rescued with WT, SA, or SE mutant of *Pkp1* (Supplementary Fig. 2F, 3B). DSG1 still shows intercellular junctional localization, however, loss of *Pkp1* does causes more diffusive staining of DSG1 in regenerated skin, suggesting potential desmosomal abnormalities.
For our immunoprecipitation analysis, the protein lysates were prepared with RIPA lysis buffer, which can efficiently dissolve protein bound protein (please see revised Material & Methods).
We have included insets in the revised Figure 7A to provide higher resolution view of Dsp staining. Consistent with DSG1 staining, Dsp localizes at intercellular junctions in the *Pkp1* KO skin, but loss of *Pkp1* leads to more diffusive localization of DSP in the cytoplasm.
All our immunofluorescence staining was carried out with sections pre-fixed in 4% paraformaldehyde (please see revised Material & Methods).

6) A recent paper (Kwa et al Journal of Biological Chemistry Vol 289 No 45 pp 31077-31087 November 7, 2014) showed involvement of RIPK4 in controlling expression of keratinocyte differentiation regulators. The current study knocks out RIPK4 in mouse skin (an important advance towards elucidating how RIPK4 regulates epidermal differentiation). However, analysis of differentiation and desmosome components (esp. Dsg1) is given short shrift. The 2014 Kwa et al. paper should be referenced, as well (a 2015 paper by the same lead author was referenced, but both are important to the current study).

Kwa et al.'s work has been cited and discussed in the revised manuscript.

7) Plakoglobin and Desmoplakin also exhibited changes in phosphorylation in the RIPK4 KO samples, an important finding that could have implications for differentiation, signaling, and cytoskeletal architecture.

We thank the reviewer for pointing this out. We have included this issue in the revised Discussion (page 16, second paragraph).

8) In the final figure the authors address the signaling pathway by which Pkp1 phosphorylation impacts epidermal differentiation, focusing on a previously reported signaling hub involving Desmoglein 1, Erbin, and SHOC2. They find an interaction between both WT Pkp1 and the phosphomimetic form of Pkp1 with SHOC2, drawing the conclusion that Pkp1-SHOC2 binding can enhance epidermal differentiation by interfering with the Ras/MAPK signaling pathway. These data are interesting but not fully fleshed out. In this regard, see comment regarding Dsg1 in point 5 above. In addition, they do not show whether Pkp1 can bind SHOC 2 or not in RIPK4 KO cells, even though they state in the discussion section that Pkp1 can associate with SHOC2 after RIPK4-mediated phosphorylation. Overall the mechanism proposed in Figure 7 could be strengthened.

We thank the reviewer for this suggestion. As described in our response to point 5, we have analyzed DSG1 localization in Pkp1 KO and rescued cells. Although Pkp1 does not directly associate with DSG1 in our biochemical assay, loss of Pkp1 leads to more diffusive staining of DSG1 in the skin.
As suggested, we also tested Pkp1 and Shoc2 binding in RIPK4 KO cells. Our new result indicates that Pkp1 interaction with Shoc2 is significantly diminished in RIPK4 null cells (Fig. 7F). Together, it strongly suggests that RIPK4/Pkp1 regulate epidermal differentiation through Shoc2 binding.

Additional comments to authors:

1) Many of the references utilized in the manuscript are dated, particularly the references associated with the introduction of epidermal differentiation and cell adhesion molecule functions.

New references have been included.

2) Language editing by a native speaker or use of a professional editing service would help to improve the text.

The revised manuscript has been proofread by an English native speaker.

3) The Pkp1-deficient cells grafted onto mice in the first portion of the study should be consistently referred to as Pkp1-KO rather than calling them Pkp1 mutant (It is my understanding that CRISPR-cas9 deleted the gene, but did not cause mutations and this could increase confusion since Pkp1 mutations in phosphorylation sites are studied later in the manuscript).
4) Figure 3b and 3c - should the figure labels read Pkp1KO+Pkp1SE rather than SD?

Corrected.

5) In the text for Figure 4 results, it is stated that Krt-5 positive cells extend to the suprabasal layers, while in the figure it says Krt-14. Which is correct?

Corrected.

6) Is there an explanation for why there is not a difference (authors say "comparable") in Ha-ras induced pErk levels in WT and Pkp1KO cells (supplementary Fig 2) while Pkp1 KO alone leads to increased pErk, the subject of Figure 7? To me it actually seems as if the KO levels may be higher in Supp. Fig. 2.

We thank the reviewer for pointing it out. We can detect a small but consistent increase in pi-Erk in the KO/Ras cells. We have revised the text accordingly.

7) The discussion section is quite broad and some parts seem more suited for an introduction or review rather than a discussion.

As suggested by multiple reviewers, we have revised the Discussion section as suggested.

Referee #3:

The authors used a well-established technology, the SILAC mouse technology coupled to high-resolution mass spectrometry, to quantify phosphoproteomic changes in keratinocyte in culture according to their state of differentiation. The goal of the study was to study how changes in the phosphoproteome regulate self-renewal and differentiation of epidermal stem and progenitor cells. Not surprisingly, the authors found that desmosomal proteins represent an enriched-group of phosphorylated proteins during differentiation. The manuscript focuses on one of them, Pkp1. Using carefully controlled experiments the authors identified the phosphorylation site in Pkp1 that is crucial for skin epidermal differentiation and the kinase responsible for the phosphorylation. They further used mouse genetic to show functionally the role of this kinase in skin differentiation and carcinogenesis. Study of Pkp1 and RIPK4 regulating differentiation is strong but the mechanism linked to tumorigenesis is less clear especially regarding to the role of SHOC2-Ras/Raf-Erk axis. Overall, this is a well-written study that tackles important questions about what control epidermal differentiation and skin tumorigenesis at the molecular level.

We thank the reviewer for appreciating the novelty of our study.

Specific major concerns essential to be addressed to support the conclusions

1) Figure 2F: This figure is very confusing and the figure legend does not explain clearly where the poorly differentiated tumor comes from. Moreover, the rational to use a6+b1+ cells, the meaning of Tgfbr2 or Ptk2 in the figure are unclear. Along these lines, the authors should discuss the work of Zanivan et al., 2013 who identify using the SILAC technology desmosomal proteins Dsg1 and Dsg2 significantly downregulated during skin differentiation and SCC progression.

We thank the reviewer for raising this issue. Actually, the same criticism was raised by reviewer 1. We have revised the text as suggested. “To test for a role of Pkp1 in skin carcinogenesis, we first explored its relative expression levels on published microarray data sets that compared transcriptomes of tumor propagating cancer cells (TPCs) isolated from moderately to poorly differentiated control and $Tgfbr2^{KO}$ SCC and well differentiated $Ptk2^{KO}$ and $Tgfbr2^{KO}/Ptk2^{KO}$ SCCs to normal skin epithelial stem and progenitor cells (Schober & Fuchs, 2011). *Pkp1* (mRNA) is consistently reduced in TPCs compared to normal skin epithelial stem and progenitor cells with lowest expression levels in highly tumorigenic $Tgfbr2^{KO}$ TPCs (Fig. 2F). These data suggest that reduced *Pkp1* expression might accelerate skin carcinogenesis and progression.”

We have cited and discussed the work of Zanivan et al. as suggested.

2) Human skin tumors are easy to obtain and authors should look if Pkp1 and Ripk4 are found downregulated according to the stage of differentiation.

We agree with the reviewer that examination of human skin tumor samples will address the potential clinical relevance of our findings. We are currently establishing collaborations with different clinical groups to collect the samples and carry out the studies. Accomplishing this test will likely reveal novel therapeutic targets and/or prognosis markers for cutaneous SCC in the future. We have included this discussion in the revised manuscript.

3) The authors should clarify in supplemental figure 3 the size differences between the WT and the exogenous Pkp1 expression and if the doublet bands correspond to proteolysis.

The exogenous Pkp1 contains triple HA tag, so it migrates slower comparing with endogenous protein in SDS-PAGE. The lower bands most likely represent proteolysis of the exogenous protein.

4) Figure 4: It will be useful to show where RIPK4 is expressed in the epidermis and confirm its loss of expression in the cKO backskin. Also it is expected that proliferation should not change in the cKO skin but this need to be shown.

We have performed additional western blots using both epidermis and dermis, and our results confirmed specific loss of *RIPK4* in the epidermis of cKO animals (Fig. 4A and supplementary Fig. 4C).

We have also examined cell proliferation as suggested. Our results show that cell proliferation is not significantly affected by RIPK4 deletion.

5) The quality of the histological staining to characterize the RIPK4 cKO SCC is poor and the analysis should be confirmed by a pathologist. The authors wrote an entire paragraph (p10, line 6) comparing the WT and RIPK4 cKO tumors but the images shown in supplemental figure 5 do not support the description. The images shown do not reflect hematoxylin and eosin staining which is usually blue, red and pink and not brown as depicted here. Hematoxylin and Eosin staining on paraffin-embedded tissues is commonly used to preserve the tissue architecture. This comment is also valid for the histology in supplemental figure 2E.

We thank the reviewer for the constructive criticism. We have re-analyzed our samples, and new staining with representative sections have been included in the revised manuscript (Supplementary Fig. 5 and 2F). We have also consulted pathologists in our dermatology department to confirm the nature of SCCs derived from the two-stage chemical carcinogenesis model.

6) Figure 5H: it is not clear on the rationale to look for expression level of RIPK4 and PKP1 in hair follicle stem cells and on the various mouse models used, similarly to figure 2F. Degree of differentiation in SCC could be analyzed on the TPA, papilloma and SCC (DMBA) models.

We thank the reviewer for pointing it out. Please see our response to Figure 2F for clarification. We have also removed the data from hair follicle stem cells as suggested.

Minor concerns that should be addressed

1) Please reference or show the data showing that 12h of calcium switch is a good time point to see early differentiation as not all readers are skin experts.

We have included an analysis of early differentiation marker expression 12 hours post calcium shift (Supplementary Fig. 1C). Our data shows initiation of early differentiation in this time point.

2) Figure 2D: it will be useful to show where Pkp1 is expressed in the epidermis here (and not in the Figure 7) and confirm its loss of expression in the KO skin graft.

Moved the figures as suggested.

3) Supplemental figure 2E: should be properly labeled as WT+V12Hras and Pkp1-KO+ V12Hras.

Changed as suggested.

4) Western blots are all lacking size markers and many of them seems overloaded (Figure 4A, Supplemental Figure 2). The quality of the western blots could be improved.

Included molecular size markers as suggested. We thank the reviewer for the suggestion. We have improved our western blots as suggested, including Fig. 4A.

5) In Figure 4 the authors described in the text the use of Krt-5 and labeled the figure with Krt-14.

Corrected as suggested.

6) p11 line 3: the authors referenced supplemental figure 5A, 5B instead of 6A, 6B.

Corrected.

7) Figure 7: age of the tissue should be mentioned.

The sections were obtained from grafted skin, which was generated from primary keratinocytes isolated from newborn pups. The skin grafts were transplanted to ~2-3 months old nude mice. We have revised the Material & Method to include this information.

Referee #4:

Lee et al. submitted a manuscript on phosphorylation of Pkp1 by RIPK4 and its role in regulation of epidermal differentiation and skin tumorigenesis. The authors performed several SILAC-based phosphoproteome analyses of differentiated and undifferentiated keratinocytes. In their dataset, several phosphorylation sites on the Pkp1 appeared to be upregulated, which motivated the authors to perform follow-up experiments linking Pkp1 phosphorylation with RIPK4 activity, epidermal differentiation and skin tumorigenesis. As requested by the Editor, and in the interest of time, I here focus solely on the proteomics part(s) of the study. Proteomics was done using state-of-the-art methodology (SILAC), equipment (Q-Exactive) and software (MaxQuant suite from the Mann lab). However, key information on the experiments is either unclear or missing. This includes:

We are grateful to the reviewer for the constructive comments on our proteomics analysis.

Major points:

• Description of the statistical treatment of significant (phospho)peptide ratios - the authors used a cutoff of 2-fold change, which is completely arbitrary; this is concerning, given the fact that the median of measured SILAC ratios is not at log2=0 (it is around log2=-0.5, see figure 1B). This means that negative ratios will be enriched in "significantly" changing proteins. The authors should rather use the "significance B" value provided by the software.

We thank the reviewer for pointing out this issue. We rechecked our SILAC phosphoproteome data (in the supplemental table), the median of normalized SILAC ratios of phosphorylated peptides is close to 0 (at log2=-0.08). We apologized that the style we presented the data in our previous Figure 1B is somewhat confusing. We made a new Figure 1B, which the interval of y axis is changed to better present the data. We chose 2-fold change as a cutoff as many other labs routinely used for SILAC experiment, such as Mann lab (Kruger et al, 2008; Zanivan et al, 2013), Andersen lab (Kristensen et al, 2012), and Choudhary lab (Satpathy et al, 2015).

• The shift towards negative ratios (see above) may be caused by technical factors, such as incomplete labeling or a large mixing error; this should be discussed in the text (and the labeling/mixing data should be submitted as supplementary material)

The question is similar to question 1. This issue is mainly caused by the way we presented Figure 1B. The labeling efficiency of heavy labeled proteins was about 98% as shown in the new Supplementary Fig. 1A, which is a reasonable condition for SILAC experiment.

• Phosphopeptide ratios should be normalized with (unmodified) protein ratios to ensure that the observed change in protein phosphorylation is not driven by gene expression (rather than kinase activity)

The SILAC ratio we presented was not normalized by protein expression. We apologize that we could not re-examine it because we no longer have the samples. However, this should not represent a significant issue as we collected the differentiated cells at very early time point (12 hours), the potential changes in protein level will be minimum. We have also confirmed the expression level of several key proteins by immunoblots, including Pkp1, which shows comparable level of proteins in both samples (supplementary Fig. 1E).

• Supplementary tables should contain information on localization probability (and significance B) and only localized sites (e.g. probability >75%) should be discussed

We added the information of localization probability into the supplemental tables, and the localization probability of all our identified phosphosites were over 0.75.

• The authors should provide annotated spectra of (at least) the three Pkp1 phosphorylation sites, demonstrating their correct localization on the peptide backbone

The annotated spectra of the Pkp1 phosphorylation sites have been provided in Supplementary Fig. 1F.

Minor points

• Brief description of the phosphopeptide enrichment protocol (or a reference) is missing

The phosphopeptide enrichment was based on a reported protocol (Mazanek et al, 2007). In brief, tryptic peptides were dissolved in loading buffer (6% TFA, 80%ACN, 1M lactic acid), and then incubated with titanium dioxide beads (Titansphere, GL Sciences, Japan) for 30min at room temperature. The titanium dioxide beads were then washed with loading buffer and wash buffer (0.5% TFA, 50%ACN). The phosphopeptides were eluted from the beads with 10% NH3H2O. Information has been included in the revised Material and Methods.

• Text should be thoroughly edited for proper use of English grammar

The revised manuscript has been proofread by a native speaker, as suggested.

It is possible that the authors observed most of these criteria, but this is not visible from the text. Until these points are fully addressed, the interpretation of proteomics results may be misleading and does not meet the standards of the EMBO J.

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2nd Editorial Decision 05 March 2017

Thank you for submitting the revised version of your manuscript. It has now been seen by all four original referees, whose comments are enclosed below.

As you will see, the referees #1 and #4 were much more critical on the revised study than referees #3 and #4, however we decided - in light of the strong support of the latter - to give you the opportunity to revise your manuscript to address the referees' points. Thus, I would like to invite you to submit a revised version of the manuscript using the link enclosed below, addressing the reviewers' comments.

Both referees #2 and #3 find that their concerns have been sufficiently addressed and are in favour of publication. However, reviewer #1 remains more critical and expresses remaining reservations related to the lack of SA/SE mouse genetics in the current study to proof causality between p-Pkp1 / Ripk4 and epidermal differentiation, which in his/her view leads to not sufficiently well supported claims and undermines the robustness of the findings (ref #1, pt.1). This referee also asks you to more clearly indicate microscopy data from mouse skin vs. skin made in the epidermal culture system (ref #1, pt.2).

Please note that in addition to the original reports, referees #2 and #3 have also provided extensive cross-comments on the issues raised by referee #1, arguing in favor of the current manuscript, stating with regards to the need of Pkp1 mouse genetics: Referee #3: 'In my point of view full KO mice can also be misleading when analyzing only one tissue (skin). The phenotype obtained can be also related to the loss of function in other cell types. In that case conditional tissue-specific KO is the best but long to obtain. The author should be more careful in their statement but I consider acceptable the use of the ex-vitro/engraftment technique.' . Referee #2: 'I also do not have a problem with the ex vivo approach. Every approach has its limitations, including full KO mice (and even using mouse systems, as human skin is very different from mouse).' .

Referee #4 states that the proteomic analyses and methods section have improved, however in his/her view still lack robustness and relevant details. Accordingly, this referee asks you to strengthen the current conclusions by carefully revising the statistics and technical documentation.

Please note that we conclude that genetic KO data on Pkp1 on the signaling involved as suggested by referee #1 is certainly of interest, but in our view beyond the scope of the current study.

Considering all of these points, we invite you to submit a revised manuscript addressing referee #1's and referee #4's comments. Along these lines, I would like to ask you in particular to

• re-consider the claims and statements made regarding the LoF experiments (Ref#1, pts.1,3)
• clearly distinguish microscopy data from mouse skin vs. skin made in the epidermal culture system (Ref #1, pt.2).
• revise and complement the proteomics statistics and technical documentation (Ref #4).

Please see below for some formal formatting issues, which need to be adjusted at re-submission.

Please submit a final revised version of the manuscript using the link enclosed below, addressing the reviewers' comments.

REFEREE REPORTS

Referee #1:

The authors of the paper entitled "Phosphorylation of Pkp1 by RIPK4 regulates epidermal differentiation and skin tumorigenesis" have made significant efforts to comprehensively tackle all the reviewers' comments.
In general, the manuscript is therefore improved - the authors have addressed all concerns relating to missing controls which needed to be included, outstanding statistical analysis, further embellishment of the material and methods section, and a complete overhaul of the Discussion section (which was criticized by at least two sets of reviewers).

However, fundamentally, the experiments shown and the conclusions presented are the same as before. The fundamental flaw of this study is that mouse genetics is not used to fully test their hypotheses. Instead of making KO and knock-in mice (e.g. Pkp1 KO, Pkp1-SA, Pkp1-SA), the authors employ an ex-vitro/engraftment technique for all their crucial experiments.

This means that although the results attained using this technique fully support their hypothesis, they cannot say for certain that phosphorylation of Pkp1 by Ripk4 is needed to ensure epidermal differentiation. This needs to be further clarified in the manuscript, so that there can be no confusion for the reader. Detailed below are a few suggestions in order to do this.

1. Relating to the Pkp1 KO data, in Figure 2

Unfortunately, the published Pkp1 KO mouse (Rietscher et al., 2016) essentially scoops the data presented in Figure 2A-DE. A genuine mouse KO is a more definitive way to show the Pkp1 is important in epidermal differentiation than the 'CRISPR followed by differentiation and grafting' technique used in this paper. This does not mean that this data is not valuable however, and should still be included. But for honesty's sake, I think this section is best reversed. First mention the published KO, what they found, and THEN introduce your data. You can say that you found the same thing using your engraftment technique - this, in a way, will actually help to validate your engraftment model in the mind of the reader.

2. Genuine mouse skin IF images vs ex-vitro "skin" made through epidermal culture system

The authors do employ mouse genetics for some of this study - the K14-Cre x Ripk4fl/fl experiment. The IF images of the skin presented in Figure 4 are essentially indistinguishable from the IF images from the epidermal culture system "skin" IF images presented everywhere else. I think the distinction is a very important one, as they are fundamentally different experiments.

Firstly, I think it would be helpful for the reader to see a schematic of the experimental protocol/outline for the epidermal differentiation/engraftment experiment. This could be placed in Figure 2, ahead of the Pkp1 experiments, for example. Secondly, all "skin" images generated from this technique must be labeled on the actual figures. It can be in white writing in any black spaces present on the IF images, or just above. The reader should be able to glance at the images and know immediately the difference between real mouse skin (Figure 4 - can also label if authors think

necessary) and ex-vitro "skin".

3. Published Ripk4 KO epidermal phenotype vs author's Ripk4 KO ex-vitro skin phenotype

The published Ripk4 KO mouse has severe hyperplasia of both the spinous and granular layers (Holland et al., 2001). In this study, the authors only see an alteration in the spinous layer, and no difference in the granular layer (last paragraph of the Discussion). What accounts for this difference?

The authors have not discussed at all the limitations of the system used in this paper (of course, why would they place doubt upon their own findings, I understand that), however I do think it is important that this issue is addressed and discussed honestly. Can the authors' really get away with using a sentence like: "In this report, we present compelling genetic evidence that loss of Pkp1 leads to significant defects in epidermal differentiation in vitro and in vivo, and its role in this process is dependent upon RIPK4-mediated phosphorylation of its N-terminal head domain."? It is important that this paper not be written as if genuine mouse knockouts were used.

In conclusion, as I stated in my first set of comments, the findings of this paper are novel and interesting. The phosphoproteomics experiments are elegant and informative. It is up to EMBO Journal to decide if experiments conducted without mouse genetics are sufficiently convincing to warrant publication by that journal.

Referee #2:

The authors have provided a thoughtful response to the previous comments, and have adequately revised figures and text. While still broad in scope, the paper is more focused and streamlined compared with the initial submission. This paper should be interesting to a broad audience.

Referee #3:

The authors have now answered all the comments I initially raised and they improved the manuscript tremendously. I therefore consider that this article should be published in EMBO journal

Referee #4:

Lee et al. have submitted a revised version of the manuscript on phosphorylation of Pkp1 by RIPK4 and its role in regulation of epidermal differentiation and skin tumorigenesis. I will here again focus mostly on the (phospho)proteomics part. While several of my points were clarified, there are still inconsistencies in the manuscript that have to be addressed:

1) distributions of SILAC ratios look better after the authors used normalized ratios; nevertheless, the cutoff of 2-fold is arbitrary and this should be clearly stated. All references to "significant" changes should be removed, as this word usually implies statistical significance.

2) for the first SILAC experiment it is not clearly stated in the text what was heavy- and what was light-labeled sample. Only after thorough reading and analysis it can be concluded that the calcium switch was done on the light cells (hence the inversed SILAC ratios in the EV1 table). Labeling should be clearly stated in the text and figure.

3) the corresponding table EV1 has improved, but it now reveals that many P-sites on Pkp1 N-terminal head domain show increased ratio: up to the residue 238, there are 13 localized P-sites of which 10 are "upregulated" and one is even highly downregulated during differentiation. For this reason the Figure 1D is incomplete and it is not clear why the authors focused only on the "prominent" S4, S120 and S143 phosphorylation events. Actually, there are higher-scoring regulated P-sites in this region and it is not clear why they weren't followed up. If the authors had any

additional evidence to disregard multiple phosphorylation events on Pkp1, or if the follow up of these sites did not lead to any result, they should state so.

4) in the SILAC comparison of the RIPK4 KO and WT cells (table EV2), only 3 out of 6 detected Pkp1 P-sites were lower than the (arbitrary) cutoff of 0.5 - given the fact that the median of the whole dataset was at 0.92, I doubt that these SILAC ratios were significant. The authors would need to prove this (e.g. by revealing the "Significance B")

5) I disagree with the authors' response on the need to normalize P-peptide data for protein expression - after 12h you will definitely see changes in protein expression. While they show that Pkp1 is not changing in the first SILAC experiment (EV Figure 1E), they do not show that the level of Pkp1 is equal in RIPK4 KO and WT cells (Figure 6 or EV6). This is essential for correct interpretation of the second SILAC experiment.

To conclude, the manuscript lacks information of the additional P-sites on PkP1 (that may imply action of another kinase on Pkp1). The second SILAC experiment, as currently presented, does not prove beyond doubt that RIPK4 acts on Pkp1. I also note that the kinome library, in which RIPK4 was identified as the kinase of interest, is poorly described - I could not find information on the completeness of this library (how many kinases are included/missing) and the screening data is missing (what were the 32P incorporation values for other kinases, how significant was the RIPK4 result etc.)

2nd Revision - authors' response 09 March 2017

Overall Comments:

We are delighted that all reviewers found that our revision has significantly improved the manuscript. Referee #1 and 4 made additional comments. We've now addressed these issues, and in doing so, have further improved the manuscript and its impact. All major changes are highlighted in the left margin. We really thank all the reviewers for all of their constructive comments!

Referee #1:

The authors of the paper entitled "Phosphorylation of Pkp1 by RIPK4 regulates epidermal differentiation and skin tumorigenesis" have made significant efforts to comprehensively tackle all the reviewers' comments.
In general, the manuscript is therefore improved - the authors have addressed all concerns relating to missing controls which needed to be included, outstanding statistical analysis, further embellishment of the material and methods section, and a complete overhaul of the Discussion section (which was criticized by at least two sets of reviewers).

We thank the reviewer for appreciating our efforts to address all the reviewers' comments in last revision.

However, fundamentally, the experiments shown and the conclusions presented are the same as before. The fundamental flaw of this study is that mouse genetics is not used to fully test their hypotheses. Instead of making KO and knock-in mice (e.g. Pkp1 KO, Pkp1-SA, Pkp1-SA), the authors employ an ex-vitro/engraftment technique for all their crucial experiments.

This means that although the results attained using this technique fully support their hypothesis, they cannot say for certain that phosphorylation of Pkp1 by Ripk4 is needed to ensure epidermal differentiation. This needs to be further clarified in the manuscript, so that there can be no confusion for the reader. Detailed below are a few suggestions in order to do this.

We thank the reviewer for pointing out this important issue. To efficiently dissect signaling cascades during epidermal differentiation, we have developed this novel experimental platform by using cultured epidermal progenitor cells and skin transplantation. It is particularly useful for studying skin stratification, as transplanted skin epidermal cells differentiate and stratify as normal skin (Fig. 2B and EV Fig. 2B, C, and D). However, we agree with the reviewer that it is important to clarify this issue and distinguish *ex vivo* results with skin transplantation and *in vivo* results with traditional

mouse genetics. We have revised the manuscript as suggested by the reviewer.

1. Relating to the Pkp1 KO data, in Figure 2

Unfortunately, the published Pkp1 KO mouse (Rietscher et al., 2016) essentially scoops the data presented in Figure 2A-DE. A genuine mouse KO is a more definitive way to show the Pkp1 is important in epidermal differentiation than the 'CRISPR followed by differentiation and grafting' technique used in this paper. This does not mean that this data is not valuable however, and should still be included. But for honesty's sake, I think this section is best reversed. First mention the published KO, what they found, and THEN introduce your data. You can say that you found the same thing using your engraftment technique - this, in a way, will actually help to validate your engraftment model in the mind of the reader.

We thank the reviewer for this suggestion. We have revised the text accordingly (page 5 last paragraph).

2. Genuine mouse skin IF images vs ex-vitro "skin" made through epidermal culture system

The authors do employ mouse genetics for some of this study - the K14-Cre x Ripk4fl/fl experiment. The IF images of the skin presented in Figure 4 are essentially indistinguishable from the IF images from the epidermal culture system "skin" IF images presented everywhere else. I think the distinction is a very important one, as they are fundamentally different experiments.

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We thank the reviewer for this suggestion. We have included a diagram that shows the procedure of skin grafting (Fig. 2D). In addition, for the IF images generated from grafted skin, we added the label “Skin graft” to the images (Fig. 2B and E, Fig. 3A, Fig. 6D, Fig. 7A, EV Fig. 2C, D, and F, EV Fig. 3B, and EV Fig. 6C and D) to distinguish them from the real mouse skin.

3. Published Ripk4 KO epidermal phenotype vs author's Ripk4 KO ex-vitro skin phenotype

The published Ripk4 KO mouse has severe hyperplasia of both the spinous and granular layers (Holland et al., 2001). In this study, the authors only see an alteration in the spinous layer, and no difference in the granular layer (last paragraph of the Discussion). What accounts for this difference?

A discussion is included in the revised manuscript (page 18, first paragraph).

The authors have not discussed at all the limitations of the system used in this paper (of course, why would they place doubt upon their own findings, I understand that), however I do think it is important that this issue is addressed and discussed honestly. Can the authors' really get away with using a sentence like: "In this report, we present compelling genetic evidence that loss of Pkp1 leads to significant defects in epidermal differentiation in vitro and in vivo, and its role in this process is dependent upon RIPK4-mediated phosphorylation of its N-terminal head domain."? It is important that this paper not be written as if genuine mouse knockouts were used.

We have included a discussion of our system in the revised manuscript (page 18, first paragraph). We have revised the particular sentence “…compelling genetic evidence…” as suggested (Page 15, second paragraph).

In conclusion, as I stated in my first set of comments, the findings of this paper are novel and interesting. The phosphoproteomics experiments are elegant and informative. It is up to EMBO Journal to decide if experiments conducted without mouse genetics are sufficiently convincing to

warrant publication by that journal.

Referee #2:

The authors have provided a thoughtful response to the previous comments, and have adequately revised figures and text. While still broad in scope, the paper is more focused and streamlined compared with the initial submission. This paper should be interesting to a broad audience.

We thank the reviewer for appreciating our efforts to address all the reviewers' comments in last revision.

Referee #3:

The authors have now answered all the comments I initially raised and they improved the manuscript tremendously. I therefore consider that this article should be published in EMBO journal

We thank the reviewer for appreciating our efforts to address all the reviewers' comments in last revision.

Referee #4:

Lee et al. have submitted a revised version of the manuscript on phosphorylation of Pkp1 by RIPK4 and its role in regulation of epidermal differentiation and skin tumorigenesis. I will here again focus mostly on the (phospho)proteomics part. While several of my points were clarified, there are still inconsistencies in the manuscript that have to be addressed:

We thank the reviewer for all the constructive comments.

1) distributions of SILAC ratios look better after the authors used normalized ratios; nevertheless, the cutoff of 2-fold is arbitrary and this should be clearly stated. All references to "significant" changes should be removed, as this word usually implies statistical significance.

We thank the reviewer for pointing out this issue. We have removed "significant" in the text (page 5).

2) for the first SILAC experiment it is not clearly stated in the text what was heavy- and what was light-labeled sample. Only after thorough reading and analysis it can be concluded that the calcium switch was done on the light cells (hence the inversed SILAC ratios in the EV1 table). Labeling should be clearly stated in the text and figure.

Revised as suggested (text page 4, and Fig. 1A).

3) the corresponding table EV1 has improved, but it now reveals that many P-sites on Pkp1 N-terminal head domain show increased ratio: up to the residue 238, there are 13 localized P-sites of which 10 are "upregulated" and one is even highly downregulated during differentiation. For this reason the Figure 1D is incomplete and it is not clear why the authors focused only on the "prominent" S4, S120 and S143 phosphorylation events. Actually, there are higher-scoring regulated P-sites in this region and it is not clear why they weren't followed up. If the authors had any additional evidence to disregard multiple phosphorylation events on Pkp1, or if the follow up of these sites did not lead to any result, they should state so.

We thank the reviewer for pointing out this issue. We focused on these three sites because our initial analysis of mutants on other sites suggests that the other sites do not significantly contribute to skin differentiation. We have revised the text to state this (Page 5 second paragraph, and Page 9 first paragraph). We have included additional discussion in the revised manuscript to address potential other kinases that phosphorylate Pkp1 head domain (page 15, last paragraph).

4) in the SILAC comparison of the RIPK4 KO and WT cells (table EV2), only 3 out of 6 detected Pkp1 P-sites were lower than the (arbitrary) cutoff of 0.5 - given the fact that the median of the whole dataset was at 0.92, I doubt that these SILAC ratios were significant. The authors would need to prove this (e.g. by revealing the "Significance B")

We agree with the reviewer that it is critical to confirm the SILAC ratio change in RIPK4 KO samples for the detected Pkp1 sites. We have manually checked the MS data of these three sites. As shown below (Figures for reviewers, Fig. 1-3), the MS1 signal intensities of these peptides are strong enough to allow a good peak area calculation. The results of our manual calculation of peak areas are consistent with the analysis of MaxQuant software. In addition, consistent with our proteomics analysis, mutagenesis and biochemical assays also confirmed that RIPK4 phosphorylate Pkp1 on these three sites (Fig. 3E-G).
As the median ratio of our whole dataset is 0.92, 2-fold change in these three sites strongly suggest that their corresponding phosphorylation is significantly changed in *RIPK4* null samples. Two fold change cutoff (or even 1.5 fold) has been widely used and reported in the leading proteomics journals (Blagoev et al, 2004; Moller et al, 2012; Wang & Huang, 2008).
As suggested by the reviewer, we also performed statistical analysis with Significance B. The P values for the three sites (S4, 120, and 143) are 0.1077, 0.2408, and 0.0088 respectively (Fig. 6C).

5) I disagree with the authors' response on the need to normalize P-peptide data for protein expression - after 12h you will definitely see changes in protein expression. While they show that Pkp1 is not changing in the first SILAC experiment (EV Figure 1E), they do not show that the level of Pkp1 is equal in RIPK4 KO and WT cells (Figure 6 or EV6). This is essential for correct interpretation of the second SILAC experiment.

We thank the reviewer for pointing it out. We have confirmed the level of Pkp1 in WT and *RIPK4* KO cells, and the immunoblots were included in the revised manuscript (EV Fig. 6C).

To conclude, the manuscript lacks information of the additional P-sites on PkP1 (that may imply action of another kinase on Pkp1). The second SILAC experiment, as currently presented, does not prove beyond doubt that RIPK4 acts on Pkp1. I also note that the kinome library, in which RIPK4 was identified as the kinase of interest, is poorly described - I could not find information on the completeness of this library (how many kinases are included/missing) and the screening data is missing (what were the 32P incorporation values for other kinases, how significant was the RIPK4 result etc.)

We thank the reviewer for the constructive comments. We have included additional discussion in the revised manuscript to address potential other kinases that phosphorylate Pkp1 head domain (page 15, last paragraph).

We have revised the Results and Method to include the information of our kinome library (Page 9, second paragraph, and Page 23, first paragraph). Our screen identified RIPK4 as a key kinase that phosphorylates Pkp1 at the three sites in the head domain (screening results shown in Fig. 3E, and phosphorylation of Pkp1 is confirmed by both in vitro and in vivo kinase analysis , Fig. 3F and G). From our screen, we did identify several other kinases that phosphorylate Pkp1, and we are very interested in pursuing their potential role in skin differentiation in the future.

References:

Blagoev B, Ong SE, Kratchmarova I, Mann M (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat Biotechnol* **22:** 1139-1145

Moller A, Xie SQ, Hosp F, Lang B, Phatnani HP, James S, Ramirez F, Collin GB, Naggert JK, Babu MM, Greenleaf AL, Selbach M, Pombo A (2012) Proteomic analysis of mitotic RNA polymerase II reveals novel interactors and association with proteins dysfunctional in disease. *Mol Cell Proteomics* **11:** M111 011767

Wang X, Huang L (2008) Identifying dynamic interactors of protein complexes by quantitative mass spectrometry. *Mol Cell Proteomics* **7:** 46-57

Figures for reviewers:

Figure 1

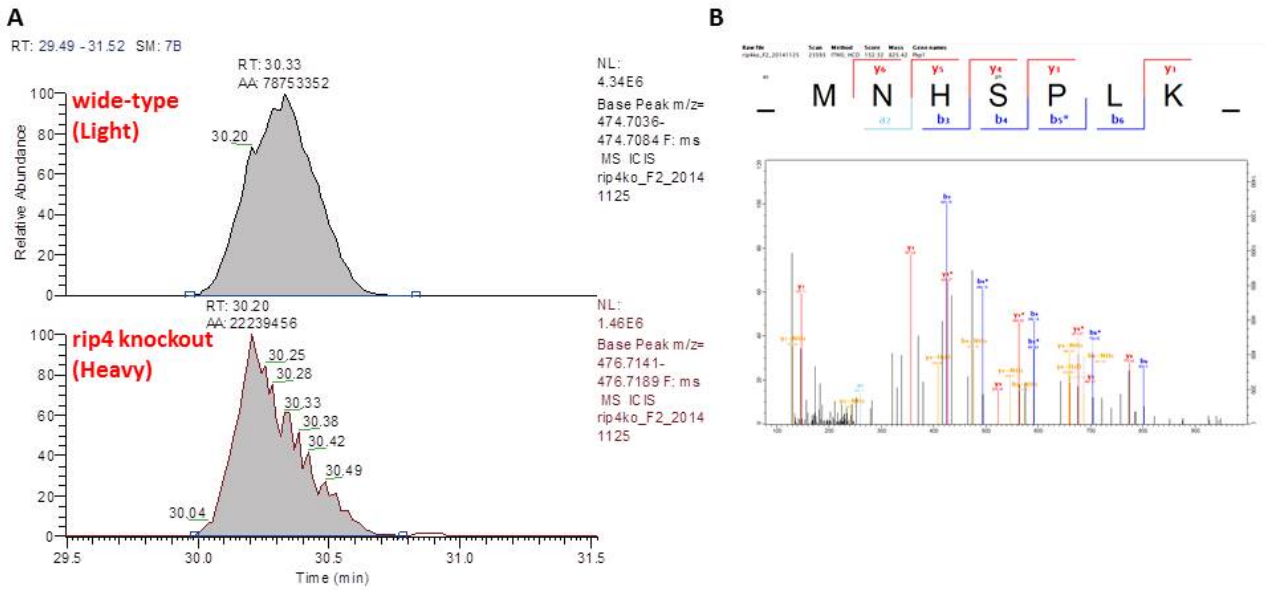

Figure 1. Peak area (left) and annotated MS/MS spectrum of phosphosite S4 (Peptide: (ac)MNHS(ph)PLK) (right). **A.** Manual checking result of MS1 peak area of phosphosite S4. Peak area was extracted in Thermo Xcalibar Qual Browser using m/z=474.7060 for wide-type (light) peptide and m/z=476.7165 for rip4 knockout (heavy) peptide. Peak area of light and heavy labeled peptide are 78753352 and 22239456 respectively. Ratio H/L by manual checking is 0.28. **B.** Best localized MS/MS spectrum of phosphosite S4 (MaxQuant).

Figure 2

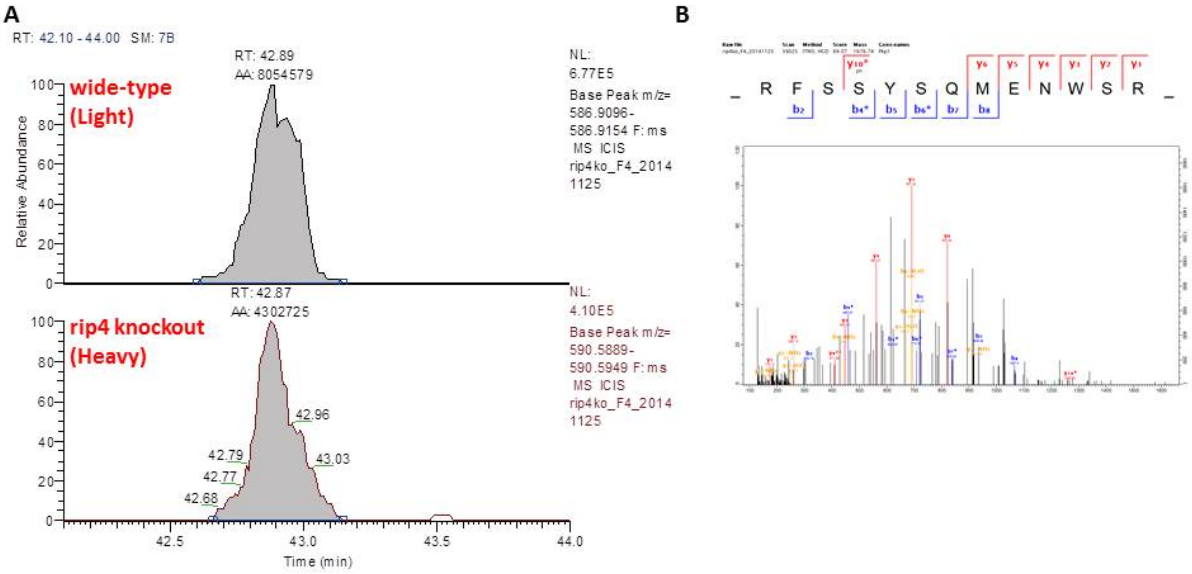

Figure 2. Peak area (left) and annotated MS/MS spectrum of phosphosite S120 (Peptide: RFSS(ph)YSQMENWSR) (right). **A.** Manual checking result of MS1 peak area of phosphosite S120. Peak area was extracted in Thermo Xcalibar Qual Browser using m/z=586.9125 for wide-type (light) peptide and m/z=590.5919 for rip4 knockout (heavy) peptide. Peak area of light and heavy labeled peptide are 8054579 and 4302725 respectively. Ratio H/L by manual checking is 0.53. **B.** Best localized MS/MS spectrum of phosphosite S120 (MaxQuant).

Figure 3

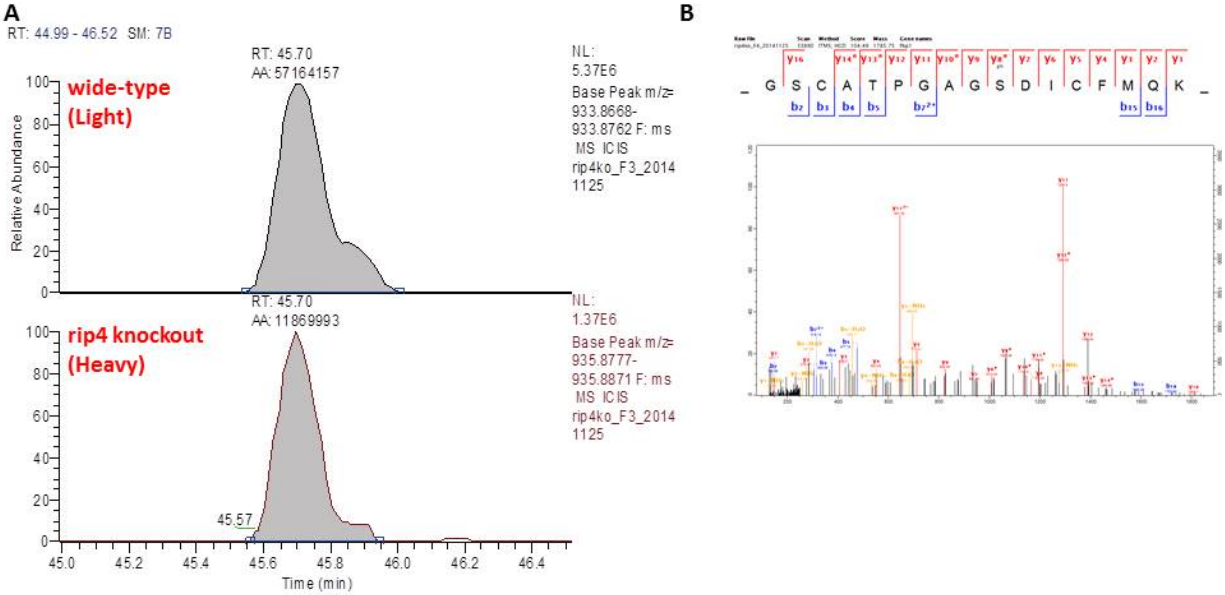

Figure 3. Peak area (left) and annotated MS/MS spectrum of phosphosite S143 (Peptide: GSCATPGAGS(ph)DICFMQK) (right). **A.** Manual checking result of MS1 peak area of phosphosite S143. Peak area was extracted in Thermo Xcalibar Qual Browser using m/z=933.8715 for wide-type (light) peptide and m/z=935.8824 for rip4 knockout (heavy) peptide. Peak area of light and heavy labeled peptide are 57164157 and 11869993 respectively. Ratio H/L by manual checking is 0.21. **B.** Best localized MS/MS spectrum of phosphosite S143 (MaxQuant).

3rd Editorial Decision 03 April 2017

Thank you for submitting the revised version of your manuscript. It has now been seen by one of the four original referees, whose comments are enclosed below.

As you will see, referee #4 has re-assessed the proteomics part of the study. This referee states that many of his concerns have been sufficiently addressed, but also points to several inconsistencies, which is his/her view remain and need to be addressed.

Thus, given the strong support from the other three referees, I would like to invite you to submit a final revised version of the manuscript using the link enclosed below, addressing this reviewers' comments.

Please note that while the point on a comprehensive representation of the kinome screen data is per well taken, we in this case do not consider including the entire data set as critically needed.

Please see below for some formal formatting issues, which need to be adjusted at re-submission.

REFEREE REPORTS

Referee #4:

The authors have addressed most of my comments, but several inconsistencies remain:

1) The figure 1D is still incomplete, as it shows only three P-sites on the N-terminus of Pkp1. The authors should indicate all identified P-sites in the figure (otherwise the figure is misleading).

2) The authors should note in the text and figure 6 legend that the reduction in the levels of Ser4 and Ser120 phosphorylation is statistically not significant.

3) The authors state in the rebuttal that their kinome screen revealed other kinases that act on Pkp1. They should provide, in form of a detailed supplementary table, the complete results of the kinome screen and state what are the other kinases that act on Pkp1, as they can also be involved in regulation of skin differentiation. Actually, I am surprised that the authors did not mention these kinases in the manuscript or checked their involvement in differentiation given the fact that there are multiple P-sites on Pkp1. I think that EMBO J should not allow partial publication of data.

3rd Revision - authors' response 03 April 2017

We are delighted that all reviewers found that our revision has significantly improved the manuscript. Referee 4 made additional comments. We've now addressed these issues, and in doing so, have further improved the manuscript and its impact. All major changes are highlighted in the left margin. We really thank all the reviewers for all of their constructive comments!

We have also addressed all formatting changes needed for the revised manuscript, including:

>> revised labeling of expanded view figures to 'Figure EV1', ...etc.

>> improved image quality and adjust contrast for Figure 7F, Figures EV1E, EV3A and EV6C.

>> adjusted figure legend of Figure 2C and show the respective western blot data in expanded view Figure EV2. Figure legends and callouts have been updated accordingly.

Referee #4:

The authors have addressed most of my comments, but several inconsistencies remain:

We thank the reviewer for all the constructive comments.

1) The figure 1D is still incomplete, as it shows only three P-sites on the N-terminus of Pkp1. The authors should indicate all identified P-sites in the figure (otherwise the figure is misleading).

Information on the other P-sites is now included in EV Fig. 1'G.

2) The authors should note in the text and figure 6 legend that the reduction in the levels of Ser4 and Ser120 phosphorylation is statistically not significant.

We revised text and Figure legend as suggested.

3) The authors state in the rebuttal that their kinome screen revealed other kinases that act on Pkp1. They should provide, in form of a detailed supplementary table, the complete results of the kinome screen and state what are the other kinases that act on Pkp1, as they can also be involved in regulation of skin differentiation. Actually, I am surprised that the authors did not mention these kinases in the manuscript or checked their involvement in differentiation given the fact that there are multiple P-sites on Pkp1. I think that EMBO J should not allow partial publication of data.

We have included additional description of our kinome analysis (Page 9) in the revised manuscript, including other potential kinases that may phosphorylate Pkp1.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xiaoyang Wu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95679R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen based on our preliminary test and prior experience with the same type of experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size for animal experiments, including skin carcinogenesis analysis, was chosen based on preliminary examination of the phenotype, and our previous experience with the same type of experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There is no sample exclusion for all the in vitro analysis. For in vivo experiments, animals that died before the end of the experiment were excluded. The exclusion criteria is pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization or blinding was used in this study.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization or blinding was used in this study.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was used in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used in this study.
5. For every figure, are statistical tests justified as appropriate?	All statistical tests are justified.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We have used multiple statistical tools to test the signifcance, as described in the manuscript.
Is there an estimate of variation within each group of data?	No.
Is the variance similar between the groups that are being statistically compared?	Yes for most of the tests. For groups with different vairations, we used Mann-Whitney U test to examine the P value.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have included the inforamtion for all the antibodies in the Material and Methods section in the revised manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used only primary mouse keratinocytes in this study. Potential contamination with mycoplasma was screened using the ATCC universal mycoplasma detection kit.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the mice were bred and maintained in the ARC (animal resource center) of the University of Chicago in accordance with institutional guidelines. Strain, gender, and other informations have been included in the Method & Material sections in the revised manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments with mice have been approved by IACUC (Institutional Animal Care and use Committee) of the University of Chicago.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	It is compliant with the guideline.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects involved.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	No human subjects involved.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No human subjects involved.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human subjects involved.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No human subjects involved.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	No human subjects involved.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	No human subjects involved.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We will deposit the proteomics following the guideline.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We will deposit the proteomics following the guideline.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	No human-related data.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Yes.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Do not have computatoinal models.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.