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Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation

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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.)

Editor: Esther Schnapp / Barbara Pauly

1st Editorial Decision

05 January 2015

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed reports on your study.

As you will see, all referees acknowledge that the data are convincing and that the study is well done and a useful contribution to the field. They only raise a few concerns, and given the limited number, I think that all of them should be addressed.

We would therefore like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. The revised manuscript should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 supplementary

figures. Please let me know if you would like to include more than 5 main figures, as we recently decided that we will also publish longer articles from now on. Can you, may be reduce the number of supplementary figures by combining the ones that relate to the same main figure? You could have more than 5 supplementary figures, but it would be good to have clearly less than 10. Shortening of the manuscript text may be made easier by combining the results and discussion section which may help to eliminate some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Please also change the reference style to the numbered EMBO reports style, which will further help to reduce the character count.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFeree REPORTS:

Referee #1:

In the present manuscript, Kouwenhoven and colleagues aimed at identifying p63 transcriptional network along human epidermal cell differentiation. To this end, the authors used ChIP sequencing, RNA sequencing and epigenomics to characterize the cluster of genes actively regulated by p63 in proliferating keratinocytes and at different time points of their differentiation. The authors report that only a subset of p63 bound regulatory elements is active as shown by the active enhancer mark H3K27ac. These histone marks in p63 bound enhancer correlate better with gene expression than the sole p63 binding. Interestingly, the authors show that p63 binding itself is not dynamic during keratinocyte differentiation. They propose that p63-cofactors such as RUNX1 and NR2 could also modulate p63 bound enhancer activity to regulate gene expression along differentiation. Overall, the experiments are very well done and the results are clear. The authors do report a very interesting description of p63 transcriptional network in epidermis that will be extremely useful in the field. The manuscript is nonetheless quite hard to read as it is even for people working on epithelial cell biology and should be rewritten to be more accessible to a broad audience. Some of the claims of the article are a bit too strong and the authors should avoid any overstatement. I think that with few modifications this study would be of interest for the audience of EMBO Report.

Major point:

The authors say that they used RNAPII occupancy at the gene body as the measurement for gene expression to study p63-regulated transcription in their analyses because Spearman correlation analysis showed a better correlation of H3K27ac levels with RNAPII occupancy at gene bodies than the mRNA abundance. This result may indicate that transcription of the genes is regulated by other factors that inhibit transcription in spite of the H3K27ac marks or point to the existence of regulation by miR. These points should be properly discussed to clarify which transcripts are indeed

regulated by p63 to orchestrate keratinocyte differentiation.

The authors highlight the genes that are positively modulated by p63 as shown by the activating histone mark H3K27ac and RNAPII binding in the gene bodies. It would be nonetheless interesting to determine which genes are repressed by p63 as they may be crucial for cell differentiation regulation as well. These genes should belong to the 50% of p63 binding sites that are not active enhancer elements. Since the repressive histone mark H3K27me3 is not found in any of the p63 binding site, it suggests that these repressions might not be epigenetic or associated to other marks such as H3K9.

The authors report that the p63 levels at the binding sites showed no tendency to correlate with nearby gene body RNAPII levels during differentiation and conclude that H3K27 occupancy at these binding sites reflects better the regulatory activity of p63 binding sites than p63 binding itself. Moreover, H3K27 occupancy also correlates well with gene expression outside p63 binding sites (p12 last paragraph). This sentence is somehow quite confusing. It suggests that p63 binding is not important per se while its role in cell differentiation is well demonstrated by gene knockdown in several studies. The authors should clarify this part of the text.

Minor point:

In the Figure 1, the authors say that H3K27me3 repressive mark was not detected in human keratinocytes but it is hard to draw any conclusion from these data as there is no peak visible. It is therefore hard to say whether the experiment just did not work.

Referee #2:

The manuscript entitled, "Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation," by Kouwenhoven et al. is an important study shedding light on the mechanisms employed by p63 during epidermal differentiation.

Importantly, the authors of the paper used primary human keratinocytes derived from patient biopsies and induced differentiation in cell culture.

Overall, the study is well done, and the data are technically sound and compelling.

I have a few comments that should be addressed prior to publication:

1. The authors should demonstrate the level of differentiation using western blot analysis of various key markers of differentiation for all the time points used in their RNA-Seq and ChIP seq analysis. These should include: K5, K14, K10, K1, filaggrin, and involucrin. This analysis should be done in addition to the RNA analysis shown in Figure 1A. This is key because complete differentiation is rarely induced in 2D culture systems.
2. The authors used an antibody that detects only alpha isoforms of p63. TAp63alpha and ΔNp63alpha. The authors completely ignore any potential contribution by TAp63alpha. Discussion on this point should be included. Additionally, western blot analysis showing the expression of p63 isoforms during the various time points should be included.
3. Some of the background p63 literature is outdated and biased. Information on the roles of p63 isoforms in differentiation are available especially using mouse models. This should be included in the Intro/Discussion to provide a complete picture of the field.
4. Many points in the Discussion are overstated and make claims that are not true. For example, the authors state, "p63 plays a regulatory role during epidermal differentiation was up to now not yet fully understood" implying that this study provides this information. While this study is important, such statements should be avoided and toned down as there are a number of published papers demonstrating that p63 regulates epidermal differentiation. These should be cited and discussed.

Referee #3:

The manuscript by Kouwenhoven et al. follows up on the identification of the genomic targets of the p63 transcription factor in human keratinocytes, previously published by the same group. The Authors use here a dynamic keratinocyte differentiation model to report the binding of p63, RNA-seq profilings and RNA Pol II locations, as well as the mapping of H3K27ac, a marker of enhancers. Correlations of binding and functions are made and a coherent view of the p63 emerges, particularly in enhancer locations.

The experiments are well performed and the conclusions, although not conceptually novel for differentiation processes, are relevant in the field. In addition, the data represent an important atlas for future genomic experiments, and for colleagues involved in studies of specific pathways. The manuscript is written in a clear and intelligible way.

For all these reasons, I support publication of the manuscript as is.

1st Revision - authors' response

11 March 2015

We like to thank you for forwarding the constructive comments of reviewers to our manuscript, entitled '***Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation***'. We have revised our manuscript according to the referees' comments, your suggestions and the manuscript guidelines of EMBO Reports.

According to your suggestions and the guidelines of EMBO Reports, we have re-written our manuscript, by combining Results and Discussions, to remove redundant text and reduce the total number of characters, and to make the manuscript more understandable for a broader readership. We have moved 'Material and methods' fully to the 'Supplementary information', to further reduce the number of characters. We believe that EMBO Reports readers can understand our manuscript without reading the 'Material and methods' part. As the result, we have reduced the total number of characters from 78,422 to 47,299 including spaces and references. However, we found it impossible to further reduce the total number of characters to below 35,000, without compromising the message conveyed in the manuscript. Since you mentioned in your decision letter that EMBO Reports will start to publish longer papers, and we discussed this in our previous e-mails, I hope that this is not a problem. In addition, we have combined several supplementary figures to make the total number of supplementary figures to five.

I also like to mention a few points regarding the statistics and source data.

1. We have included the specification of all data quantification, such as 'n' for the number of experiments and tests for the P value calculation. All the information has been added to the appropriate part of the manuscript.
2. According to the guideline, we need to have the exact number for P values, using 'P value = X' instead of 'P value < X'. However, in our manuscript, we have used 'P value < X' under two circumstances. One of them is when analysis algorithms are used, often the parameters used are P values. In this case we can only state the parameters as 'P value < X'. Another situation is that for several analyses, we have combined different pairs of comparison, and presented the P value using the highest P value, and therefore 'P value < X'. In this case, the exact P values are presented either in the supplementary tables or in the source data.
3. We also include all source material of the analyzed data and western blots.

We thank you for your time and consideration and look forward to your favorable answer.

Detailed responses to reviewers' comments:

Referee #1:

Overall, the experiments are very well done and the results are clear. The authors do report a very interesting description of p63 transcriptional network in epidermis that will be extremely useful in

the field. The manuscript is nonetheless quite hard to read as it is even for people working on epithelial cell biology and should be rewritten to be more accessible to a broad audience. Some of the claims of the article are a bit too strong and the authors should avoid any overstatement. I think that with few modifications this study would be of interest for the audience of EMBO Report.

We have considerably re-written the manuscript to make the manuscript understandable for a broader audience. We have also revised some of the statements to avoid overstatement (see below).

Major point:

The authors say that they used RNAPII occupancy at the gene body as the measurement for gene expression to study p63-regulated transcription in their analyses because Spearman correlation analysis showed a better correlation of H3K27ac levels with RNAPII occupancy at gene bodies than the mRNA abundance. This result may indicate that transcription of the genes is regulated by other factors that inhibit transcription in spite of the H3K27ac marks or point to the existence of regulation by miR. These points should be properly discussed to clarify which transcripts are indeed regulated by p63 to orchestrate keratinocyte differentiation.

We agree with the referee. The observation that a better correlation of H3K27ac levels with RNAPII occupancy at gene bodies than the mRNA abundance may indeed indicate that the transcripts are regulated by other mechanisms such as miRNA regulation and mRNA stability. We have added these discussions (P9).

The authors highlight the genes that are positively modulated by p63 as shown by the activating histone mark H3K27ac and RNAPII binding in the gene bodies. It would be nonetheless interesting to determine which genes are repressed by p63 as they may be crucial for cell differentiation regulation as well. These genes should belong to the 50% of p63 binding sites that are not active enhancer elements. Since the repressive histone mark H3K27me3 is not found in any of the p63 binding site, it suggests that these repressions might not be epigenetic or associated to other marks such as H3K9.

Our strategy cannot differentiate whether p63 functions as an activator or a repressor. The activity of p63 binding sites is determined by H3K27ac occupancy, and absence of H3K27ac at p63 binding sites (inactive p63 binding sites) could be due to different mechanisms. In addition to H3K27me3, we have examined the repressive H3K9me marks as requested and included these data in our new Fig 2A. Similar to H3K27me3, these repression marks are not co-localized with p63 binding sites (Fig. 2A). Therefore, it is unlikely that lack of activity at p63 binding sites is through the polycomb complex repression (H3K27me3) or through heterochromatin formation (H3K9me3). We hypothesize that p63 may need co-activators to activate transcription. We have added these discussions in the text (P13).

The authors report that the p63 levels at the binding sites showed no tendency to correlate with nearby gene body RNAPII levels during differentiation and conclude that H3K27 occupancy at these binding sites reflects better the regulatory activity of p63 binding sites than p63 binding itself. Moreover, H3K27 occupancy also correlates well with gene expression outside p63 binding sites (p12 last paragraph). This sentence is somehow quite confusing. It suggests that p63 binding is not important per se while its role in cell differentiation is well demonstrated by gene knockdown in several studies. The authors should clarify this part of the text.

Indeed, our observations stated as 'H3K27 occupancy at these binding sites reflects better the regulatory activity of p63 binding sites than p63 binding itself', and 'H3K27 occupancy also correlates well with gene expression in general apart from at p63 binding sites' suggest that p63 binding alone is not sufficient for gene transcription in epidermal cells and not all epidermal genes are dependent on p63. This does not contradict to the important role of p63 in cell differentiation. It rather suggests that, in addition to p63, other factors are critically involved in epidermal differentiation. We have discussed these points in the text (P11-12).

Minor point:

In the Figure1, the authors say that H3K27me3 repressive mark was not detected in human keratinocytes but it is hard to draw any conclusion from these data as there is no peak visible. It is therefore hard to say whether the experiment just did not work.

Data of the H3K27me3 mark are from the ENCODE consortium. H3K27me3 signals are not co-localized with p63 binding sites but are present at inactive gene promoters. We have included these data in Supplementary Fig 5.

Referee #2:

Overall, the study is well done, and the data are technically sound and compelling.

I have a few comments that should be addressed prior to publication:

1. The authors should demonstrate the level of differentiation using western blot analysis of various key markers of differentiation for all the time points used in their RNA-Seq and ChIP seq analysis. These should include: K5, K14, K10, K1, filaggrin, and involucrin. This analysis should be done in addition to the RNA analysis shown in Figure 1A. This is key because complete differentiation is rarely induced in 2D culture systems.

Advanced differentiation is indeed rarely observed (or reported) in submerged 2D cultures. The model that we used here allows advance differentiation as shown in previous publications. This has now been mentioned and referenced in the text (P6). As requested by the referee, we have performed additional western blot analyses to demonstrate expression at the protein level for undifferentiated basal cells (KRT14), early differentiation (KRT10), advanced differentiation (involucrin) and very late differentiation (loricrin). These new data are included in Supplementary Fig 1. We trust that we have convinced the referee that, despite being a 2D culture system, our model mimics the situation in normal human epidermis.

2. The authors used an antibody that detects only alpha isoforms of p63. TAp63alpha and ΔNp63alpha. The authors completely ignore any potential contribution by TAp63alpha. Discussion on this point should be included. Additionally, western blot analysis showing the expression of p63 isoforms during the various time points should be included.

We do not observe the TAp63 isoform in any of our RNA-seq, RNAPII ChIP-seq and western blotting analyses. We observed a band with a higher molecular weight in the western blot analysis using the p63 H129 antibody (alfa-specific isoform) and the intensity of this band increases during differentiation (source file, western blots). This band could be mistaken as the N-terminal longer TA isoform. However this band is absent in the western blot using the p63 4A4 antibody (recognizes all isoforms), and therefore cannot be the TA isoform. Our data show that the deltaN-p63 is the major isoform, if not the only isoform, in epidermal cells, and this conclusion has been confirmed by a recent RNA-seq report in mouse epidermis and by many isoform specific mouse studies, discussed in the manuscript (P6).

3. Some of the background p63 literature is outdated and biased. Information on the roles of p63 isoforms in differentiation are available especially using mouse models. This should be included in the Intro/Discussion to provide a complete picture of the field.

We have included the following papers on isoform-specific mouse models (below).

· Chakravarti, D., Su, X., Cho, M.S., Bui, N.H., Coarfa, C., Venkatanarayan, A., Benham, A.L., Flores Gonzalez, R.E., Alana, J., Xiao, W., Leung, M.L., Vin, H., Chan, I.L., Aquino, A., Muller, N., Wang, H., Cooney, A.J., Parker-Thornburg, J., Tsai, K.Y., Gunaratne, P.H. & Flores, E.R. Induced multipotency in adult keratinocytes through down-regulation of DeltaNp63 or DGCR8. *Proceedings of the National Academy of Sciences of the United States of America* 111, E572-581 (2014).

· Romano, R.A., Smalley, K., Magraw, C., Serna, V.A., Kurita, T., Raghavan, S. & Sinha, S. DeltaNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. *Development* 139, 772-782 (2012).

· Pignon, J.C., Grisanzio, C., Geng, Y., Song, J., Shivdasani, R.A. & Signoretti, S. p63-expressing cells are the stem cells of developing prostate, bladder, and colorectal epithelia. *Proceedings of the National Academy of Sciences of the United States of America* 110, 8105-8110 (2013).

· Suh EK et al (2006) p63 protects the female germ line during meiotic arrest. *Nature* **444**: 624-628

4. Many points in the Discussion are overstated and make claims that are not true. For example, the authors state, "p63 plays a regulatory role during epidermal differentiation was up to now not yet fully understood" implying that this study provides this information. While this study is important, such statements should be avoided and toned down as there are a number of published papers demonstrating that p63 regulates epidermal differentiation. These should be cited and discussed.

We have rephrased these statements.

Referee #3:

The manuscript by Kouwenhoven et al. follows up on the identification of the genomic targets of the p63 transcription factor in human keratinocytes, previously published by the same group. The Authors use here a dynamic keratinocyte differentiation model to report the binding of p63, RNA-seq profilings and RNA Pol II locations, as well as the mapping of H3K27ac, a marker of enhancers. Correlations of binding and functions are made and a coherent view of the p63 emerges, particularly in enhancer locations.

The experiments are well performed and the conclusions, although not conceptually novel for differentiation processes, are relevant in the field. In addition, the data represent an important atlas for future genomic experiments, and for colleagues involved in studies of specific pathways. The manuscript is written in a clear and intelligible way.

For all these reasons, I support publication of the manuscript as is.

No further comments.

Correspondence - editor

8 April 2015

Many thanks for the submission of the revised version of your manuscript to our editorial office. Since my colleague Esther Schnapp, who is the handling editor of your paper, is currently out of the office I am writing to you to avoid unnecessary delays.

I am happy to tell you that the referee who assessed the revised version of the study now fully supports the publication of it in our journal. When going through the files myself I did, however, notice that the manuscript still does not contain a materials and methods section, as was also noted by Esther in her previous letter to you.

I certainly realize that this stems from the fact that the study was originally submitted to The EMBO Journal as a longer article and that you had to significantly shorten it for EMBO reports.

I should mention that we have now started to publish two different types of articles, one is still called 'scientific report' and can contain up to five main figures. This type of article may contain a short M&M section in the main text and the rest in the supplementary information. In this article type, the results and discussion section is combined.

The other type of article is referred to as 'article' and in addition to being able to accommodate more than five figures, it can also contain more characters. However, in this article type, all M&Ms have to be displayed in the main text (i.e. no supplementary M&Ms are allowed) and the results and discussion sections have to be separate.

At the moment, your manuscript is a hybrid between the two article types and I would kindly ask you to choose as what kind you want it to be published. Personally, I think it is better suited as a full 'article' (rather than a short report) and if you agree to have it published as such, I would kindly ask

you to move the M&M section in its entirety to the main text and split up the R&D section.

I am fully aware that this basically means moving back closer to the format of the original article and I fully understand that this must seem like extra and unnecessary work to you (and for that I am profoundly sorry). Unfortunately, when Esther was discussing the shortening of your paper with you, we didn't have the option of a full 'article' yet. If you prefer the 'scientific report' option, you may keep the R&D section combined but I would need to ask you to incorporate the main materials and methods in the main text and display some peripheral methods as supplementary information. The number of characters should in this case at least not be any higher than it is now, i.e. in order to accommodate the M&M section, you would need to shorten some other parts further.

Please do let me know what you prefer.

Again, I apologize for this inconvenience during this transition period and thank you very much for your cooperation.

Please do get in touch with me if you require further information and help in preparing the final version of your manuscript.

REFEREE REPORT:

Referee #2

The authors have completely addressed our previous concerns.

Correspondence - authors

17 April 2015

Here I send you our re-formatted version of our manuscript, including the final main text and the final supplementary file. I hope that everything is clear. If there is any question, please let me know.

2nd Editorial Decision

20 April 2015

Many thanks for submitting the re-formatted version of your manuscript to EMBO reports. I think it was worth the effort and I appreciate your cooperation in this matter.

Below, please find the official acceptance letter with more information on the next steps. If you have any further questions, please do not hesitate to contact me, or my colleague Esther, who will be back in the office later this week.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.