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Long non-coding RNA uc.291 controls epithelial differentiation by interfering with the ACTL6A/BAF complex

Emanuele Panatta, Anna Maria Lena, Mara Mancini, Artem Smirnov, Alberto Marini, Riccardo Delli Ponti, Teresa Botta-Orfila, Gian Gaetano Tartaglia, Alessandro Mauriello, Xinna Zhang, George A Calin, Gerry Melino and Eleonora Candi

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(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 16 August 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires major revisions to before publication in EMBO reports. As the reports are below, and I think all points need to be addressed in a revised manuscript and/or in a detailed rebuttal letter, I will not further detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your 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.

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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that the corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account!

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 paper entitled "Long noncoding RNA uc.291 controls epithelial differentiation by interfering with ACTL6A/BAF complex" the authors characterize a lncRNA transcribed from ultraconserved regions and suggest his role in epidermal differentiation.

The manuscript is well written and easy to follow and the biological observation is potentially interesting, however a few points need to be addressed.

Major points:

-The figure legends say that the authors performed RT-qPCR, but the figures are full of gel pictures. Now if the authors performed qPCR in real-time, the endpoint detection of qPCR products in a gel is uninformative and should be removed. I would like to see instead the control WB for ChIP and RIPs that are missing and not optional!

-For RIPs at least another nuclear unrelated transcript should be checked as a negative control.

- Page 4: "Amplification of the C10orf11 flanking exons were below the RT-qPCR detection levels during keratinocyte differentiation, indicating that uc.291 is transcribed independently from C10orf11 mRNA". The authors should provide this piece of data in a qPCR graph showing side by side amplification of uc.291 and the surrounding exons of the other gene.
Also, to prove that this is a transcriptional, rather than an RNA processing event, the authors should demonstrate the presence of an independent promoter for uc.291 (a survey of the encode data would help here together with the H3K27ac ChIP used throughout the paper).

-The authors suggest in the discussion that uc.291 may be implicated in squamous cell carcinoma. Did they check the TCGA database for loss of its expression and/or mutations in this non-coding transcript?
-Does ACTL6a contain an RNA-binding domain? Do I interpret correctly the graph in figure 5d, if I say that the dash indicates 1 consensus sequence (a linear binding motif) in uc.291?
Connected to this, looking at the last figure it is not clear to me what is the exact function of uc.291. This point should be addressed in the discussion. As far as I understood the authors suggest that uc.291 is sponging ACTL6a away from its target genes, is that correct?
Why the other 543 ACTL6a targets are not affected then? Are there differences in the binding of ACTL6a to different target genes? If this is a sponging mechanism, isn't it a bit bizarre that you have only one binding site for ACTL6a on uc.291?

-In the discussion the authors underline the fact that many other components of the complex were identified as putative interactors of uc.291 in the protein array. However, in the Result section the authors explain that they failed to validate at least another of the candidates in the same complex. Therefore, I would not emphasize the presence of the other candidates unless a validation is provided for them by RNA pulldown or RIP.

Minor points:

-The use of abbreviations in the figures makes them difficult to interpret at a first glance.
-Page 5, first sentence of the new paragraph: I assume that the meaning was "To gain further information on the role of uc.291 during differentiation, the profile of protein-coding RNA in differentiating keratinocytes uc.291 -depleted was performed".
-A cartoon explaining what to expect at different timepoints in terms of markers and morphology would help the interpretation of the differentiation experiments

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Referee #2:

In this study, Panatta and colleagues show that a lncRNA (uc.291) plays a role in human epidermal differentiation by modulating the binding of ACTL6A to the promoter of epidermal differentiation genes. Using a microarray approach, they first identified uc.291 upregulated during epidermal differentiation. When they knocked down uc.291, they observed increased proliferation and compromised expression of epidermal differentiation. Next, they identified uc.291 interacts with ACTL6A, a chromatin modifier that plays a role in governing epidermal differentiation by competing with BRM/BRG1. They showed that uc.291 KD resulted in persistence binding of ACTL6A on the promoter of LOR, FLG and LCE1B and competing against the binding of BRM/BRG1 to the same promoter. Overall, this is an interesting study that provides new insights into the role of lncRNA in epidermal differentiation and further links its function to a chromatin modifier. I have these suggestions:

1. In Figure 1 and Figures S1-3, they identified uc.291 as a nuclear lncRNA located within the intronic region of C10orf11. To confirm the existence of this transcription unit and this transcript, they should mine existing histone markers such as H3K4me3 together with H3K36me3 (define the transcription unit) and perform Northern blot for the full-length transcript. In addition, since they relied heavily on siRNA knockdown approach, they should validate the knockdown by either Northern or in situ. Their current in situ results are pretty weak, which can be improved if they can perform the in situ under the KD condition.

2. In Figure 2, they showed that uc.291 downregulation led to increased cell proliferation and

compromised expression of terminal differentiation genes. They did not specifically state whether it was under proliferative or differentiating conditions for the cell cycle test (my best guess was it's the proliferative condition). However, it is not clear why the loss of uc.291 can lead to increased proliferation since it should not be highly expressed in the proliferating keratinocytes. In addition, in their microarray data (Table S2), when they profiled differentially expressed genes in uc.291 KD, differentiated keratinocytes, there are no signature of altered cell cycle. They should investigate why the KD of uc.291 can lead to a phenotype in the proliferating cells. Figure 2A should include the percentage of cells in G0/1 and G2/M, in addition to S phase. Figure 2E/G should use a basal marker such as Krt5/14 or a suprabasal mark such as Krt10 to co-stain with Ki67 and p63.

3. In Figure 3, they studied how the loss of uc.291 compromised epidermal differentiation. The staining in Figure 3C was not convincing. Most signals for LOR and FLG in SCR appear to be from stratum corneum. They should co-stain with Krt10 with these markers to confirm. In addition, have they observed any defects in proliferation in differentiated cells when uc. 291 is knocked down? Images in Figure S5 are not very clear. I am not sure whether these data point to delayed differentiation.

4. In Figure 5 and Figure S5, they identified uc.291 binding to ACTL6A. These are difficult experiments and based on their description, it appears to have a lot of candidates that can bind to in vitro transcribed uc.291 - even top 10% results gave them 1,540 candidates! To confirm the binding, they could use a mutant uc.291 e.g. by deleting the predicted binding fragment (Fig. 5D) and testing whether it still binds to ACTL6A. Figure 5E is not very informative as any RNA can fold into some type of structures.

5. To functionally link uc.291 and ACTL6A in epidermal differentiation, they could test whether KD ACTL6A in the absence of uc.291 can recover the compromised expression of LOR, FLG and LCE1B, in addition to the bioinformatic analysis in Figure 7A. They should also calculate the possibility for the overlapping genes in Figure 7A as only a small portion of each dataset actually overlapped.

Referee #3:

Emanuele Panatta and co-authors report uc.291 as a new non-coding RNA involved in the regulation of epidermal differentiation. Using RNA- and chromatin immunoprecipitations, the authors demonstrate that uc.291 binding to the ACTL6A allows BAF complex binding to the DNA. The remodeling activity of BAF complex is known to elicit chromatin changes to promote the expression of differentiation genes in keratinocytes. Authors demonstrate that suppression of uc.291 inhibits differentiation and promotes the progenitor/undifferentiated state of the cells. Moreover, the authors show the transcriptional mechanism for suppressed differentiation by confirming that upon uc.291 depletion, ACTL6A is bound to the differentiation gene promoters and inhibits BAF complex, thereby preventing the activation of differentiation genes. Importantly, part of this study is done in human keratinocytes grown 3D epidermal equivalents, which closely correspond to the in vivo state of differentiating epidermis. The manuscript can be accepted for publication after the following revisions.

Major:

1. High calcium-induced 2D differentiation assays do not recapitulate the in vivo process of keratinocyte differentiation. The role of uc.291 during differentiation has to be confirmed by inducing differentiation by growing primary keratinocytes to a high confluency with a consequent withdrawal of growth factors from the medium (Kouwenhoven EMBO Rep 2015).

2. More experiments would be required to confirm the authors conclusion that "altogether, these results strongly indicate that uc.291 is required for regulating the switch between proliferation and differentiation and to allow the expression of genes required for terminal differentiation". The data presented in manuscript demonstrate a significant delay in late differentiation but not in the induction of the process overall (as the authors themselves point out in the discussion of the results of Fig. 3). The conclusion has to be corrected, or supported by additional experiments with the

knock-out of uc.291 in keratinocytes, followed by a failure to induce differentiation in 2D confluent cultures and 3D epidermal equivalents.

3. It is not clear why the authors chose uc.291 from the microarray: For example, uc.262 and uc.283 have much lower FDR, and uc.262 fold-change is much higher than of uc.291. Microarray results need to be presented as a heat map, and validation and candidate selection strategy have to be clarified in the text and supported by the relevant data (instead of a descriptive and incomplete flow-chart in Fig. 1A).

Minor:

1. ACTL6A needs to be introduced/explained before it is mentioned in the abstract.
2. It is unclear why the NC control is missing in FISH in Fig. 1F (even though used in situ hybridization in Fig. 1D).
3. Fig. 4B needs a better legend. It is unclear why two very similar GO analyses are demonstrated with different values on the X axis.
4. Fig. 5A: Does it shows only the interactome of the genes expressed in the epidermis? Please, make it clear in the legend.
5. Fig. 5E is too small, it is impossible to read the numbers.

1st Revision - authors' response 7 October 2019

REVIEWER 1

In the paper entitled "Long noncoding RNA uc.291 controls epithelial differentiation by interfering with ACTL6A/BAF complex" the authors characterize a lncRNA transcribed from ultraconserved regions and suggest his role in epidermal differentiation. The manuscript is well written and easy to follow and the biological observation is potentially interesting, however a few points need to be addressed.

Reply: We thank the referee for the positive comments.

1) *-The figure legends say that the authors performed RT-qPCR, but the figures are full of gel pictures. Now if the authors performed qPCR in real-time, the endpoint detection of qPCR products in a gel is uninformative and should be removed. I would like to see instead the control WB for ChIP and RIPs that are missing and not optional!*

Reply: We thank the referee for the opportunity to clarify this point. The gel pictures in Fig 5A-B shows ChIP experiments, indeed here the gel images represent the PCR-end point to better visualize the strength of the results obtained. As requested to avoid confusion, we have amended the figure legend. In addition, to perform the experiments shown in Fig 5A-B and Fig 5D we have used well characterized ChIP-grade antibodies (see Methods section, Bao X et al, 2013; Ye Z et al 2016; Degenhardt T et al, 2009; Liz Z et al 2012; Bedadala GR et al 2010); therefore, we did not perform WB to control IP also to avoid to lose precious immune-precipitated chromatin-associated material. As negative control we have used IgG. Please, note that experiment shown in Fig 5A reproduces data already published in proliferating keratinocytes for ACTL6A, BRM and BRG1 (Bao X et al, Cell Stem Cell 2013) using the same antibodies.

As requested by the referee, we have performed the control WBs for the RIP experiment (Fig 4C-D). New experiments (RNA-CLIP, Fig 4D) were also done to better validate uc.291 interactors (see also point 3) of the REVIEWER-1. The result obtained by RIP was also confirmed using RNA-CLIP (crosslinking and immune-precipitation) experiments for ACTL6A. We have also screened other subunits of the BAF complex among the top 20 interactors (Fig 4A). We confirmed the interaction of uc.291 with ACTL6A and find interaction for SMARCC2. Although, due to the cross-linking step, we are not able to discriminate among direct and indirect interaction. Nevertheless, the results shown in Fig 4C-D nicely validate the data obtained using the protoarray.

2) -*For RIPs at least another nuclear unrelated transcript should be checked as a negative control.*
Reply: As requested by the referee, we included as negative control for the RNA RIP an additional nuclear unrelated transcript. Since nuclear transcripts specific for keratinocytes differentiation have not been yet characterized, we selected a specific trancript, FAM83-AS1, described by Bao et al 2013 RNAseq experiment. FAM84-AS1 is expressed both in proliferating and differentiating keratinocytes, as evaluated by RNAseq and RT-qPCR (Fig S7A-B). RIP/RNA-CLIP experiments showed a negative result (Fig S7A-B) confirming that, unlike uc.291, FAM83-AS1 does not interact with ACTL6A. These new data are now included in the "Results" section and in supplementary Fig S7.

3) - *Page 4: "Amplification of the C10orf11 flanking exons were below the RT-qPCR detection levels during keratinocyte differentiation, indicating that uc.291 is transcribed independently from C10orf11 mRNA". The authors should provide this piece of data in a qPCR graph showing side by side amplification of uc.291 and the surrounding exons of the other gene. Also, to prove that this is a transcriptional, rather than an RNA processing event, the authors should demonstrate the presence of an independent promoter for uc.291 (a survey of the encode data would help here together with the H3K27ac ChIP used throughout the paper).*
Reply: We agree with the referee that this point has to be clarified. As requested, we included new data showing that during calcium-induced differentiation (1-9 days of treatment) the expression pattern of uc.291 transcript increases while the expression of fragments amplified by RT-qPCR corresponding to exon 2-3 and exon 6-7 of C10orf11 (known as LRMDA) (Fig 1H) decrease (Fig 1I), demonstrating that uc.291 is transcribed independently from LRMDA mRNA. The latter, also demonstrates that uc.291 is not generated by an RNA processing event. Analysis of the H3K27ac ChIP performed in differentiating keratinocyes (Kouwenhoven et al. *Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation* EMBO Rep 2015), reveals the presence of several H3K27 peaks in proximity of uc.291, one that is differentiation-specific in located around 30Kb. Additional experiments, behind the interest of this article, are needed to demonstrate that this is indeed an enhancer element for uc.291. Furthermore, due to the fact that uc.291 is a novel transcript, not yet annotated, it is not possible to predict the uc.291 promoter region. Further expensive and time-consuming studies are needed (ie. perform a de novo RNA-seq assembly using 3-9 days differentiated keratinocytes) to asses this specific referee point. With the current knowledge, we can say that uc.291 is an independent transcript. This is also specified in the "Results" section.

4) -*The authors suggest in the discussion that uc.291 may be implicated in squamous cell carcinoma. Did they check the TCGA database for loss of its expression and/or mutations in this non-coding transcript?*
Reply: We agree with the referee that this is am important point to address. We have checked the TCGA data base for uc.291 expression. However, since this is a pro-differentiation transcript and squamous cell carcinoma are mostly de-differentiated, we did not get interesting information due to the low expression. The FPKMs were to low to get significant statistic evaluation. For these reasons we explored the expression of uc.291 in other skin pathologies and found that it is modulated in psoriasis. This was assessed both using bioinformatic approaches and also by in situ-hybridization of human tissue biopsies taken from normal skin and psoriatic skin and by RT-qPCR evaluation of a small cohort of patients (n=7). Below are the preliminary unpublished data on uc.291 and psoriasis, showing the involvement of uc.291, ACTL6A/BAF complex in modulating the differentiation abnormalities seen in this inflammatory skin disease. We hypothesised that the simultaneous up-regulation of uc.291 and ACTL6A in non-lesional and lesional psoriatic skin is an attempt to prevent the differentiation defects observed in psoriasis. Further investigation are currently on going in our laboratory.
[Figures for referees not shown.]

5) *Does ACTL6a contain an RNA-binding domain? Do I interpret correctly the graph in figure 5d, if I say that the dash indicates 1 consensus sequence (a linear binding motif) in uc.291? Connected to this, looking at the last figure it is not clear to me what is the exact function of uc.291. This point should be addressed in the discussion. As far as I understood the authors suggest that uc.291 is sponging ACTL6a away from its target genes, is that correct? Why the other 543 ACTL6a targets are not affected then? Are there differences in the binding of ACTL6a to different target genes? If this is a sponging mechanism, isn't it a bit bizarre that you have only one binding site for ACTL6a on uc.291?*

Reply: We agree with the referee that we need to clarify this points. According to RBDP, ACTL6A does not contain a canonic RNA binding domain. However, as it was shown by the experiments of Hentze et al 2018 and Van Nostrand et al. 2017, proteins can bind the RNA even in the absence of a binding domain. Since eCLIP data are yet not available for ACTL6A, in our case we should recall on predictions using the *Global Score* algorithm. The *Global Score* algorithm also shows good performances on the arrays, as reported in the Supplementary Fig S6D. The F-B score was employed to select different groups of high-affinity (top ranked) and low-affinity (bottom ranked) interactions reported in the array: 1) RBP, 2) chromatin-related and 3) unfiltered (i.e., without selecting by category). In all cases, *Global Score* performances increase from poor signal (top/bottom 200) to strong signal (top/bottom 10) interactions, showing AUCs that raise from 0.60 to 0.80 (Figure below, also reported in Fig S6D). The line in Fig 4D shows the predicted binding interface between ACTL6a and uc.291. The length of the line reflects the actual length of the predicted binding region, which also falls in a highly-structured region of the uc.291 secondary structure profile. These aspects raised by the referee are now better clarified in the "Results" and "Discussion" section.

6) -*In the discussion the authors underline the fact that many other components of the complex were identified as putative interactors of uc.291 in the protein array. However, in the Result section the authors explain that they failed to validate at least another of the candidates in the same complex. Therefore, I would not emphasize the presence of the other candidates unless a validation is provided for them by RNA pulldown or RIP.*
Reply: We agree with the referee that this point need to be clarified. We have performed additional RNA-CLIP experiments testing interaction also for the other subunits of the BAF complex among the top 20 interactors (SMARCCE1, SMARCC2 and SMARCD3), we were able to validate the interaction also with SMARCC2 (Fig 4C). Due to the cross-linking step, we are not able to discriminate among direct and indirect interaction, yet we can say that RIP and RNA-CLIP techniques both verify uc.291 association to ACTL6A. These novel findings are now included in the "Results" section.

7) -*The use of abbreviations in the figures makes them difficult to interpret at a first glance.*
Reply: Following the referee suggestions we have careful revised the use of abbreviation in the Figures. We double checked that all the abbreviation used are clearly specified in the figure's legend to facilitate the readers.

8) -*Page 5, first sentence of the new paragraph: I assume that the meaning was "To gain further information on the role of uc.291 during differentiation, the profile of protein-coding RNA in differentiating keratinocytes uc.291 -depleted was performed".*
Reply: We thank the referee for raising this point. We have corrected the sentenced as suggested.

9) *A cartoon explaining what to expect at different timepoints in terms of markers and morphology would help the interpretation of the differentiation experiments*
Reply: As requested by the referee we included a cartoon in Fig 2 to summarize the alteration seen during differentiation in 3D skin equivalents and in WBs upon si-uc.291 silencing.

REVIEWER 2

In this study, Panatta and colleagues show that a lncRNA (uc.291) plays a role in human epidermal differentiation by modulating the binding of ACTL6A to the promoter of epidermal differentiation genes. Using a microarray approach, they first identified uc.291 upregulated during epidermal differentiation. When they knocked down uc.291, they observed increased proliferation and compromised expression of epidermal differentiation. Next, they identified uc.291 interacts with ACTL6A, a chromatin modifier that plays a role in governing epidermal differentiation by competing with BRM/BRG1. They showed that uc.291 KD resulted in persistence binding of ACTL6A on the promoter of LOR, FLG and LCE1B and competing against the binding of BRM/BRG1 to the same promoter. Overall, this is an interesting study that provides new insights into the role of lncRNA in epidermal differentiation and further links its function to a chromatin modifier. I have these suggestions:
Reply: We thank the referee for the positive comments.

1) *In Figure 1 and Figures S1-3, they identified uc.291 as a nuclear lncRNA located within the intronic region of C10orf11. To confirm the existence of this transcription unit and this transcript, they should mine existing histone markers such as H3K4me3 together with H3IK36me3 (define the transcription unit) and perform Northern blot for the full-length transcript In addition, since they relied heavily on siRNA knockdown approach, they should validate the knockdown by either Northern or in situ Their current in situ results are pretty weak, which can be improved if they can perform the in situ under the KD condition situ.*

Reply: We agree with the referee that this is an important point. Analysis of the existing histone markers including H3K4me3 and H3IK36me3 in human terminal differentiated kerayinocytes (6-9 days terminal differentiated keratinocytes) are not available, making impossible to perform this analysis. The only analysis possible, done in keratinocytes growth in culture conditions comparable with our experiments, was on the ChIP-seq experiment performed in differentiating human keratinocytes for the enhancer marker H3K27ac (Kouwenhoven et al. *Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation* EMBO Rep 2015), which indicates the presence of few H3K27 peaks in proximity of uc.291 (around 20Kb). Furthermore, due to the fact that uc.291 is a novel transcript, not yet annotated, it is not possible to predict the uc.291 promoter region. Further studies are needed (ie. perform a de novo RNA-seq assembly using 3-9 days terminal differentiated keratinocytes deriving from different donors) to asses this specific referee point. Interestingly, we observed that uc.291 is strongly modulated in patients affected by psoriasis, an inflammatory skin disorders. Here, as possible compensatory mechanism (studies are currently on going in our laboratory), the expression of uc.291 increases in psoriatic lesion as compared to normal skin [Figures for referees not shown.].

At the moment we are collaborating with Prof Swindell WR (Ohio University), who performed RNAseq on human psoriastic lesion (Swindell et al, Front Immunol. 2018), to perform an *ad hoc* analysis to fish out sequences matching uc.291 from existing fastq sequence files. To summarise, with the current knowledge, it is not possible to further answer the referee question.
We agree with the referee that we need to provide further evidences to test uc.291 siRNA(1) and siRNA 2) specificity. To this aim we have included new data indicating that the siRNA sequences used are high specific for uc.291, being able to knock-down uc.291 expression in A253 cancer cells. For technical reason, we could not perform the experiment in primary keratinocytes. FISH experiments performed after si-uc.291 does not reveal signal in contrast to scramble transfected cells, further confirming that the siRNA sequences identified are specific for uc.291 (Fig 1J-K).

2) *In Figure 2, they showed that uc.291 downregulation led to increased cell proliferation and compromised expression of terminal differentiation genes. They did not specifically state whether it was under proliferative or differentiating conditions for the cell cycle test (my best guess was it's the proliferative condition). However, it is not clear why the loss of uc.291 can lead to increased proliferation since it should not be highly expressed in the proliferating keratinocytes. In addition, in their microarray data (Table S2), when they profiled differentially expressed genes in uc.291 KD, differentiated keratinocytes, there are no signature of altered cell cycle. They should investigate why the KD of uc.291 can lead to a phenotype in the proliferating cells. Figure 2A should include the percentage of cells in G0/1 and G2/M, in addition to S phase. Figure 2E/G should use a basal marker such as Krt5/14 or a suprabasal mark such as Krt10 to co-stain with Ki67 and p63.*

Reply: We agree with the referee that the role of uc.291 in proliferating keratinocytes is not clear. For this reason we have omitted part of the Fig 2 (the part related to the *in vitro* keratinocytes proliferation), among the main figures in this revised version. We believe, however, that the effects of uc.291 silencing in 3D skin equivalents is interesting and in line with the abnormalities demonstrated upon uc.291 silencing. This is evident in the 3D skin equivalent experiments, in which we observed an increase of basal layer marker DNp63 expression (Fig 2D-F) and Ki67 signal (Fig S5). We think that this is possibly due to an incomplete/unbalanced terminal differentiation process in absence of uc.291, that with an unknown mechanisms, influences the proliferating compartment of the epidermis. A scheme of the markers variation has been included in Fig 2G (as also requested by referee 1) to facilitate the out-come of the uc.291 depletion in 3D skin equivalents. We agree with the referee that additional information and experiments will be needed to address the role of uc.291 in proliferation, accordingly we have modified this point in “Results” and “Discussion” sections.

3) *In Figure 3, they studied how the loss of uc.291 compromised epidermal differentiation. The staining in Figure 3C was not convincing. Most signals for LOR and FLG in SCR appear to be from stratum corneum. They should co-stain with Krt10 with these markers to confirm. In addition, have they observed any defects in proliferation in differentiated cells when uc. 291 is knocked down? Images in Figure S5 are not very clear. I am not sure whether these data point to delayed differentiation.*

Reply:

To study the effect of the loss od uc.291 during differentiation we decided to use both 2D keratinocytes cultures and 3D skin equivalents. Using both systems si-uc.291 strongly affects expression of late differentiation markers including loricrin and filaggrin, see Fig 2A-B (RT-qPCR and WB) and Fig 2C (confocal staining of 3D skin). Interestingly, K10 expression, spinous layer marker, is not affected (see Fig 2B, WB, and Fig 2C, con-focal staining of 3D skin). To better present the results we have included magnification of confocal images for locricrin. Now is clear that in control (SCR) loricrin anti-body stains not only the cornified layer but also the granulaus layer cells. The signal is strongly reduced in si-uc.291 silenced 3D skin. Similar description for filaggrin. As consequence of the defect in terminal differentiation, the keratinocytes of the basal layer results in expressing high level of DNp63, a basal layer marker (Fig 2D-F) and Ki67 (Fig S5).

4) *In Figure 5 and Figure S5, they identified uc.291 binding to ACTL6A. These are difficult experiments and based on their description, it appears to have a lot of candidates that can bind to in vitro transcribed uc.291 - even top 10% results gave them 1,540 candidates! To confirm the binding, they could use a mutant uc.291 e.g. by deleting the predicted binding fragment (Fig. 5D) and testing whether it still binds to ACTL6A. Figure 5E is not very informative as any RNA can fold into some type of structures.*

Reply: To select the candidates, we used a procedural filtering approach based on the data coming from the arrays. The 1,540 candidates are not our final candidates, but just a step in the filtering procedure. The final list of candidates is only of 40 proteins, and ACTL6A is the third candidate of the ranked list. Our filtering computational procedure narrowed down few candidates between thousands of proteins. If it is true that the RNA can fold into multiple structures, it is also true that a more "probable" native structure can be narrowed down using computational approaches. Moreover, the combination of experimental constraints, such as SHAPE, into thermodynamics-based software such as *RNAstructure* are able to provide structures with up to 90% accuracies on crystals (Low and Weeks 2010, Hajdin et al., 2013). The *CROSS* algorithm has comparable performances to SHAPE data on the crystals, also showing an improvement in the performances when applied as constraints inside *RNAstructure* (Delli Ponti et al., 2017). Combining *CROSS* with the search of the minimum free energy structure from *RNAstructure*, we could select a highly-probable native structure. Even if the RNA can exist in multiple conformations, using our approach we are confident to have selected one the most representative structures.

5) *To functionally link uc.291 and ACTL6A in epidermal differentiation, they could test whether KD ACTL6A in the absence of uc.291 can recover the compromised expression of LOR, FLG and LCE1B, in addition to the bioinformatic analysis in Figure 7A. They should also calculate the possibility for the overlapping genes in Figure 7A as only a small portion of each dataset actually overlapped.*

Reply: The rescue experiment suggested by the referee in not possible to perform, indeed knock-down of ACTL6A will allow the BAF complex to positively act on the expression of LOR, FIL, INV (see also ChIP experiments in Fig 5), in this case, the presence or the absence of uc.291 will be ineffective. Therefore, the experiment suggested will not demonstrate the link between ACTLA and uc.291. Concerning the possibility that by chance the genes among the two lists overlapped (Fig 6A), we would like to remind that the role of ACTL6A as suppressor of differentiation has been investigated in deep by Bao X et al 2013. The common genes shown in Fig 6A are inversely correlated and not overlapping, meaning that genes that are down-regulated by ACTL6A knock-down are up-regulated upon si-uc.291. The inverse correlation among them functionally demonstrated the negative regulation of uc.291 in controlling ACTL6A functions.

REVIEWER 3

Emanuele Panatta and co-authors report uc.291 as a new non-coding RNA involved in the regulation of epidermal differentiation. Using RNA- and chromatin immunoprecipitations, the authors

demonstrate that uc.291 binding to the ACTL6A allows BAF complex binding to the DNA. The remodeling activity of BAF complex is known to elicit chromatin changes to promote the expression of differentiation genes in keratinocytes. Authors demonstrate that suppression of uc.291 inhibits differentiation and promotes the progenitor/undifferentiated state of the cells. Moreover, the authors show the transcriptional mechanism for suppressed differentiation by confirming that upon uc.291 depletion, ACTL6A is bound to the differentiation gene promoters and inhibits BAF complex, thereby preventing the activation of differentiation genes. Importantly, part of this study is done in human keratinocytes grown 3D epidermal equivalents, which closely correspond to the in vivo state of differentiating epidermis. The manuscript can be accepted for publication after the following revisions.
Reply: We thank the referee for the positive comments.

1) *High calcium-induced 2D differentiation assays do not recapitulate the in vivo process of keratinocyte differentiation. The role of uc.291 during differentiation has to be confirmed by inducing differentiation by growing primary keratinocytes to a high confluency with a consequent withdrawal of growth factors from the medium (Kouwenhoven EMBO Rep 2015).*
Reply: Following referre's suggestion we have induced keratinocyte differentiation using (a) high cell conflence with withdraw of growth factors (following Kouwenhoven EMBO Rep 2015 conditions) and (b) a modified version of Howard Green medium (E-MEM medium; Rheinward&Green, Cell 1975) and compared uc.291 expression in calcium-induced differentiation. As indicated in supplementary Fig S5A, uc.291 increases its expression in all the three methods used, in parallel to the expression of late differentiation markers (K10, loricrin and LCE1B), confirming that its up-regulation during differentiation is not dependent by the differentiation-stimuli used.

2) *More experiments would be required to confirm the authors conclusion that "altogether, these results strongly indicate that uc.291 is required for regulating the switch between proliferation and differentiation and to allow the expression of genes required for terminal differentiation". The data presented in manuscript demonstrate a significant delay in late differentiation but not in the induction of the process overall (as the authors themselves point out in the discussion of the results of Fig. 3). The conclusion has to be corrected, or supported by additional experiments with the knock-out of uc.291 in keratinocytes, followed by a failure to induce differentiation in 2D confluent cultures and 3D epidermal equivalents.*
Reply: We agree with the referee that we have over interpreted our results in this specific case. Following referee suggestion we have rephrased this part and we carefully check the conclusions in order to avoid overstatements.

3) *It is not clear why the authors chose uc.291 from the microarray: For example, uc.262 and uc.283 have much lower FDR, and uc.262 fold-change is much higher than of uc.291. Microarray results need to be presented as a heat map, and validation and candidate selection strategy have to be clarified in the text and supported by the relevant data (instead of a descriptive and incomplete flow-chart in Fig. 1A).*
Reply: We agree with the referee that we need to clarify this point. The microarray to detect the expression of uc.RNAs was performed using primary keratinocytes extracted by a single donor grown in proliferating and differentiating conditions. We selected uc.291 because its expression levels were consistent when we have repeated the validation experiments by RT-qPRC using keratinocytes isolated by different donors (Fig S1B-C). Indeed, Fig S1C represents the average of expression different uc.RNAs (uc.36, uc,88 and uc.291) of 5 different human keratinocytes donors. We have excluded ucRNA, such us uc.262 and uc 283, whose expression was variable when validated in different (5) donors. This point is now clarified in the Fig S1B-C legends and in the "Results" section.

4) ACTL6A needs to be introduced/explained before it is mentioned in the abstract.
Reply: We modified the text following the referee suggestion.

5) It is unclear why the NC control is missing in FISH in Fig. 1F (even though used in situ hybridization in Fig. 1D).

Reply: Unlike in situ hybridization, the FISH procedure does not require a scramble sequence to use as negative control, because the probes are a pool of many sequences able to hybridise the target transcript. In order to address the referee 1 concern about uc.291 siRNAs specificity, we have also performed FISH upon uc.291 silencing. Results shown in Fig 1K are an additional evidence that the FISH probes used are specific.

6) Fig. 4B needs a better legend. It is unclear why two very similar GO analyses are demonstrated with different values on the X axis.
Reply: We agree with the referee that the two GO terms are not needed. We have now kept the GO term (log10(P)) shown in Fig 3D.

7) Fig. 5A: Does it shows only the interactome of the genes expressed in the epidermis? Please, make it clear in the legend.
Reply: As requested by the referee, we have now included this information in the legend.

8) Fig. 5E is too small, it is impossible to read the numbers.
Reply: As requested by the referee we have included a bigger version of the putative structure of uc.291 in supplementary figures.

REFERENCES:

- Bao X *et al*. ACTL6a enforces the epidermal progenitor state by suppressing SWI/SNF-dependent induction of KLF4. *Cell Stem Cell*. 12(2):193-203 (2013).
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-Li Z *et al.* Foxa2 and H2A.Z mediate nucleosome depletion during embryonic stem cell differentiation. *Cell* 151:1608-16 (2012).
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-Delli Ponti *et al*., A high-throughput approach to profile RNA structure. *Nucleic Acid Research* 45(5):e35 (2017).
-Van Nostrand EL, *et al.* .Robust, Cost-Effective Profiling of RNA Binding Protein Targets with Single-end Enhanced Crosslinking and Immunoprecipitation (seCLIP). *Methods Mol Biol*. 1648:177-200 (2017).
-Hentze MW, Castello A, Schwarzl T, Preiss T. A brave new world of RNA-binding proteins. Nat Rev Mol Cell Biol. 2018 May;19(5):327-341.

2nd Editorial Decision 5 December 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all three referees have remaining points and suggestions to improve the manuscript I ask you to address in a final revised version, either by adding data or text changes, and/or in a detailed point-by-point-response (in case you feel points have already been adequately addressed during the previous revision). Please provide a detailed point-by-point-response to these points in any case.

Further, I have these editorial requests:

- Please add a 'the' to the title:
Long noncoding RNA uc.291 controls epithelial differentiation by interfering with the

ACTL6A/BAF complex

- Please provide the abstract written throughout in present tense.

- The supplementary material needs to be presented differently. You can submit up to 5 figures as Expanded View Figures. Presently, there are 8 supplementary figures. Thus, please choose 5 of these to be presented as EV figures. Please follow the nomenclature Figure EV1, Figure EV2 etc., and use this for the naming of the figures and for their call outs. The figure legend for these should be included into the main manuscript document file in a section called 'Expanded View Figure Legends' after the main Figure Legends section.

- Additional Supplementary material should be supplied as a single pdf file called Appendix. The Appendix should have page numbers and needs to include a table of contents (TOC) on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

- There seem to be 4 supplementary tables. I think it would be best to move three of these into the Appendix (Tables S1, S3 and S4). Please put these after the figures into the Appendix, including their legends, and using the nomenclature Appendix Table Sx. Please use these names for all the call outs in the manuscript text.

- Table S2 is a dataset. Please name this file Dataset EV1, provide a legend on the first TAB of the excel sheet, and change the call outs in the manuscript file.

- Please remove all legends regarding items shown in the Appendix from the main manuscript text. These need to be included next to the item in the Appendix pdf file.

- In all the figure legends, please carefully check that all bars and error bars are defined, e.g. mean +/- SD. Also please define the number of replicates as well as their nature (i.e. technical or biological), and the statistical method employed.

- In Fig S3B, please move up the text '1Kb DNA lad'. I presently covers part of the image.

- Please move all methods information to the main text.

- It seems there is no call out for panel 3E. Please check.

- Please remove the abbreviation section and define each abbreviation upon its first mention in the manuscript text.

- Please add a proper conflict of interest statement (COI) to the manuscript text, after the acknowledgements.

- As they are significantly cropped, please provide the source data for the Western Blot images. 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. Please submit the source data (scans of entire gels or blots) together with the final revised manuscript. Please include size markers for the scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- It seem the author Xinna Zhang is missing from the author contributions. Please add. Further, Alberto Marini and Alessandro Mauriello seem to show up there both as AM. Please use abbreviations to distinguish their contributions.

- Please enter the funding information into our system upon resubmission.

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

Finally, Gerry Melino is indicated in our system as co-corresponding author. However, the title page of the manuscript mentions only one corresponding author. Please check. If Melino is indeed co-corresponding author, he is required to supply an ORCID ID for upon submission of the revised manuscript. Please find instructions on how to link his ORCID ID to his account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

REFEREE REPORTS

Referee #1:

The paper entitled "Long noncoding RNA uc.291 controls epithelial differentiation by interfering with ACTL6A/BAF complex" from Panatta and colleagues, has been extensively revised and improved. However I still have minor comments and questions:

1. Page 4 "Notably, the primary uc.291 transcript has the same orientation of its hosted gene"
2. Page 5 " independently from (of) differentiation stimuli used in vitro" also the different stimuli used could be spelled out in the main text explaining the differences
3. In figure 5D it looked like the acetylation at LOR locus is the least affected, while this is the most affected gene in terms of expression (Fig.1a-b), can the authors comment about it?

Referee #2:

In the revised manuscript, the authors have improved their studies. However, I am not convinced that they have sufficiently addressed an essential issue - the identify of uc.291 noncoding RNA. Based on the original paper that described uc.291 (Calin et al., 2007), it's relatively highly expressed in normal lymphocytes. And their in situ data (Fig. 1D and 1K) suggest that uc.291 is also abundantly expressed in normal epithelial cells of the skin and cancer cells, respectively. Thus, the expression of uc.291 is quite widespread and should be easily detectable in these cells. It is well known that histone H3K4me3 universally marks the promoter and TSS region of Pol II transcripts and has been extensively profiled by ENCODE projects. They should easily identify suitable cell lines with existing H3K4me3 ChIP-seq data to validate at least the unique transcription start site of uc.291 within the intron. Furthermore, it is straightforward to perform H3K4me3 and RNA-seq in keratinocytes and unequivocally identify this transcript if Northern blot is not possible. This study has the potential to be the first paper that comprehensively examines uc.291 in human cells. I believe it's imperative for the authors to convincingly demonstrate the existence of uc.291.

Referee #3:

The authors of the reviewed manuscript identified lincRNA uc.291 as a new regulator of the terminal differentiation of keratinocytes in cultured conditions and in human epidemis. The authors presented an adequate number of experiments to confirm that uc.291 interacts with an ATP-dependent nucleosome remodelling complex subunit ACTL6A, which blocks pro-differentiation processes such as EDC gene expression. The depletion of uc.291 in differentiating conditions

resulted in enhanced ACTL6A binding to loricrin, fillagrin, and late cornified envelope protein 1B, whereas the binding of BRM/BRG1 remodeling complex to the promoters of these genes was lost, possibly leading to a change of histone modifications. Thus, a decrease in H3K27ac observed upon depletion of uc.291 may explain the dramatic loss of mRNA and protein of LOR, FIL and LCE1B, even though the link between the recruitment of acetyltransferases and uc.291 function may be indirect. The direct interaction between Uc.291 and ACTL6A can explain changes in expression of 39 common genes; however, the interesting question for further research remains in the mechanism of expression of the rest 349 uc.291-regulated mRNAs that change expression in the ACTL6a-independent manner.

2nd Revision - authors' response 17 December 2019

REVIEWER 1

The paper entitled "Long noncoding RNA uc.291 controls epithelial differentiation by interfering with ACTL6A/BAF complex" from Panatta and colleagues, has been extensively revised and improved.
Reply: We thank the referee for the positive comment.

1) *Page 4 "Notably, the primary uc.291 transcript has the same orientation of its hosted gene"*
Reply: As requested by the referee, we have modified the sentence indicated.

2) *Page 5 " independently from (of) differentiation stimuli used in vitro" also the different stimuli used could be spelled out in the main text explaining the differences*
Reply: As requested by the referee, we have corrected the sentences and spelled out the different stimuli used.

3) *In figure 5D it looked like the acetylation at LOR locus is the least affected, while this is the most affected gene in terms of expression (Fig.1a-b), can the authors comment about it?*
Reply: We thank the referee for the detailed observation. We believe there is not a clearly defined correlation between % of messenger RNA reduction and % of specific histone modification reduction (in this case H3K27ac). Furthermore, from the biological point of view, the contribution of multiple histone modification, occurring in chromatin opening, is not the same for all the genes.

REVIEWER 2

1) *In the revised manuscript, the authors have improved their studies. However, I am not convinced that they have sufficiently addressed an essential issue - the identity of uc.291 noncoding RNA. Based on the original paper that described uc.291 (Calin et al., 2007), it's relatively highly expressed in normal lymphocytes. And their in situ data (Fig. 1D and 1K) suggest that uc.291 is also abundantly expressed in normal epithelial cells of the skin and cancer cells, respectively. Thus, the expression of uc.291 is quite widespread and should be easily detectable in these cells.*
Reply: We thank the referee for this comment. Indeed, as highlighted by the referee, the relative expression of uc.291, detected by PCR, is higher in normal lymphocytes respect to cancer cells (Calin *et al.* 2007). Nonetheless, it is only a relative evaluation which does not tell much about the absolute expression level. Unfortunately, neither the *in situ* hybridization (ISH) is a quantitative technique, therefore only the tissue localisation but not expression level can be assessed by ISH.

2) It is well known that histone H3K4me3 universally marks the promoter and TSS region of Pol II transcripts and has been extensively profiled by ENCODE projects. They should easily identify suitable cell lines with existing H3K4me3 ChIP-seq data to validate at least the unique transcription start site of uc.291 within the intron.
Reply: We thank the referee for this suggestion. We entirely agree with the referee that identification of the proper promoter and TSS is of pivotal importance while describing a new transcript. As we demonstrate in our manuscript, uc.291 expression is restricted to differentiated keratinocytes. Only few ChIP-seq experiments (for H3K4me3, H3K27ac, Pol2, etc) in these particular conditions are publicly available in ENCODE and GEO databases. We have analyzed

these data to assess the chromatin state in the uc.291 locus. Nevertheless, we were unable to identify any clear promoter region, only an enhancer region as indicated in the rebuttal letter. This is probably due to a very low expression level of uc.291. Indeed, comprehensive analysis of lncRNAs expression from RNAseq indicated that in a good percentage it is likely to have *false negative* results when applying strict cut off of expression (i.e. 21% of lncRNAs are not detected in any tissue and 11% are only detected in a single tissue using an RPKM threshold greater than 0.1; Derrien T et al., Genome Research 2012). As matter of fact, none of the articles published on T-UCs so far rely on ENCODE or RNA-seq datasets analysis to identify the T-UC transcript (for example see references Liz J et al., Cancer Cell 2014; Plosky BS Mol Cell 2014; Vannini I et al., Nat Commun 2017; Feng J et al., Gene&Dev 2006), instead more sensible techniques such as RTqPCR, array, ISH, FISH are used. Furthermore, we do not have evidences that u.291 is poly-adenylated. This strongly limits the unequivocally identification of uc.291 transcript in keratinocytes or in epithelial tissue since most of the RNA-seq datasets available rely on poly-A enrichment step. However, we aim to perform a detailed ChIP-qPCR analysis of putative promoter region in our future work which is outside the scope of current study.

3) *Furthermore, it is straightforward to perform H3K4me3 and RNA-seq in keratinocytes and unequivocally identify this transcript if Northern blot is not possible. This study has the potential to be the first paper that comprehensively examines uc.291 in human cells. I believe it's imperative for the authors to convincingly demonstrate the existence of uc.291.*
Reply: We thank the referee for recognizing the potential of our article. We plan to carry out a *de-novo* RNA-seq assembly using total RNA extracted from differentiated keratinocytes in our future work. Unfortunately, this experiment is time consuming and expensive, therefore at the moment the full characterization of the uc.291 transcript is beyond of the scope of current study.

REVIEWER 3

The authors of the reviewed manuscript identified lincRNA uc.291 as a new regulator of the terminal differentiation of keratinocytes in cultured conditions and in human epidemis. The authors presented an adequate number of experiments to confirm that uc.291 interacts with an ATP-dependent nucleosome remodelling complex subunit ACTL6A, which blocks pro-differentiation processes such as EDC gene expression. The depletion of uc.291 in differentiating conditions resulted in enhanced ACTL6A binding to loricrin, fillagrin, and late cornified envelope protein 1B, whereas the binding of BRM/BRG1 remodeling complex to the promoters of these genes was lost, possibly leading to a change of histone modifications. Thus, a decrease in H3K27ac observed upon depletion of uc.291 may explain the dramatic loss of mRNA and protein of LOR, FIL and LCE1B, even though the link between the recruitment of acetyltransferases and uc.291 function may be indirect. The direct interaction between Uc.291 and ACTL6A can explain changes in expression of 39 common genes; however, the interesting question for further research remains in the mechanism of expression of the rest 349 uc.291-regulated mRNAs that change expression in the ACTL6a-independent manner.

Reply: We thank the referee for the positive comments.

3rd Editorial Decision 20 December 2019

Thank you for the submission of your revised manuscript to our editorial offices. I now went through your revised manuscript and the point-by-point response, and I consider the remaining referee concerns as adequately addressed.

However, before we can proceed with formal acceptance, some points regarding statistics need to be addressed:

- In the diagrams of Figs. 1G and 3I you show error bars and statistical testing, although you indicate in the legend that only two technical replicates are shown. Thus, statistical testing does not make much sense here (with two replicates). Please show these data without statistics, by showing the two dataset separated (e.g. two bars for SS and AS).

- In general, please show separate data points/bars if n=2, and not combined data with error bars (i.e. in 1G, 1I, 1J and 3I). This is much more transparent, and illustrates better the data.

- In contrast, could statistical testing be done in Figs. 1C, 1F (for all panels), 1K, 3B and 4C/D, were n=3?

- Finally, we need information on statistics and replicates (n, technical, biological) also for the EV figures. Please go through the EV legends and make sure that bars and error bars are defined throughout, that the number of replicates as well as their nature, i.e. technical or biological, is indicated, as well as the statistical test. This needs to be done similar as for the main figure legends. Moreover, if n=3 or bigger, please provide statistical testing. If n=2, please show the two datasets separated (see above).

Otherwise, this looks fine. Please let me know if you have any questions regarding the revision.

3rd Revision - authors' response 23 December 2019

In the present version of the manuscript we have addressed all the points raised by the editor regarding the statistics of the data presented, in detail:

1) Thanks for point out these discrepancies. For Fig 3I and Fig 1G we have indicated the data without statistic, showing two bars for the two experiments performed.
2) We corrected the figure legends indicating the right replicate numbers (n=3 minimum) for Fig1I, 1J.
3) We have now included the statistical analysis for Fig 1C, 1F, 1K, 3B and 4C/D, were n=3.
4) We have provided information on statistics and replicates in the figure legends and in the EV figures

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: ELEONORA CANDI
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2018-46734V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 χ2 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	No statistical method was used to determine sample size. The sample size was sufficient to yield high statistical significance.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
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 was applied.
For animal studies, include a statement about randomization even if no randomization was used.	n/a
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.	n/a
4.b. For animal studies, include a statement about blinding even if no blinding was done	n/a
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	No estimate of variation was performed.
Is the variance similar between the groups that are being statistically compared?	No variances were calculated.

C- Reagents

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://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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 provided the required information.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the used cell lines were purchased from ATCC and used within 15 passages.

* 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.	n/a
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
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.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All samples were utilized with the approval (Protocol No. 130/18) of the institutional review board of University Hospital "Policlinico Tor Vergata" (Rome, Italy)
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.	The samples from this study were utilized prior patient consent.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
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.	n/a
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.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Gene array data: Gene Expression Omnibus GSE103890
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).	n/a
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).	n/a
21. 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.	n/a

G- Dual use research of concern

22. 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.	n/a