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reports

IκBα deficiency imposes a fetal phenotype to intestinal stem cells

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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 3 December 2019

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

As you will see, both referees think that the findings are of high interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not 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/oor 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 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 me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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 should have page numbers and needs to include a table of content 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.

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4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. See:
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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843
(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We 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. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) 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, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please format the references according to our journal style. See:
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Finally, please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines:
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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 this study, Marruecos et al investigate the role of IkappaB-alpha in intestinal stem cells. They identify a subset of cells that present nuclear IkappaB-alpha. Using genetic knockouts they associate the phonotype of deficiency with a fetal signature. Finally, they associate this fetal phenotype with a problem in PRC2-function.

Overall, this is an interesting study identfying a previously uncharacterised fundtion of IkappaB-alpha. In particular nucelar IkappaB-alpha. The presented data is of very good quality and boradly supports the claims of the authors.

However, the weakest part of the study is PRC2 connection. Even though, there is association of the

genes identified to be occupied by IkappaB-alpha and RNA regulated, there is little mechanistic evidence for the role of PRC2 in this. It is mostly a statitically probablility. So, analysis of the order of events, which comes first, PCR2 and then IkappaB, or the other way around, is necessary. Also: Does IkappaB physically associate with PRC2 components, and is this required for activity? Also controls of PRC2 component levels would be required.

The organoid studies are interesting, but some controls are missing. The phenotype is similar to that of APC mutation. Is APC affected, either in level or activity? Similar, is beta-catenin activity altered?

Finally, how much of this is NF-kappaB-dependent is not clear. It is surprising that no overt activation of NF-kappaB is detected.

On a minor point, it would be ideal to have single channel images with scale bars on the IF data. It is hard to make out nuclear areas with the images provided.

Referee #2:

Summary:
1. Does this manuscript report a single key finding? YES
Loss of Ikb causes intestinal stem cells to revert to a fetal-like phenotype
2. Is the reported work of significance YES
3. Is it of general interest to the molecular biology community? NO
The work is of interest to researchers in the fields of regeneration, stem cell biology and intestinal tumorigenesis.
4. Is the single major finding robustly documented using independent lines of experimental evidence - NO. The report is mostly based on Ikb knockout mice that have survived beyond the critical stage where most die (p7). Therefore, most data are based on a highly selected population. However, it may not take too much experimentation to generate independent lines of evidence.

Report:
In this manuscript, the authors demonstrate that phosphorylated IkB is present in the nucleus of intestinal stem cells and that loss of this gene causes these cells to revert to a fetal-like phenotype/gene expression profile. They also demonstrate that nuclear IkB is lost during acute damage of the intestine and reappears in regenerating areas. Based on these data, they conclude that nuclear IkB allows intestinal stem cells to convert from a fetal like state to an adult state during development and that loss of nuclear IkB may play a role in tissue regeneration by allowing cells to revert to a fetal, proliferative state. They provide some evidence that this occurs through altering the PRC2 complex and thus, methylation, although this is secondary to the main conclusion.

I found the manuscript to be of potential interest. It was hard to robustly review, as many details on data acquisition, data analysis and statistics were lacking from both the figure legends and methods. Below I have listed (based on the data presented) what I believe to be the main strengths and weaknesses.

Strengths: The authors reveal a novel role for IkB in the intestine that builds on recent data demonstrating that tissue damage causes intestinal stem cells to revert to a fetal state. Fuller understanding of this reversion may help develop strategies to accelerate wound healing in this and other tissues. Therefore, the finding in the manuscript, that IkB may play a role, could be of extreme interest to the field. Although the conclusions are mostly based on a knockout mouse model, they do present data demonstrating loss of nuclear IkB in damaged tissue, that is re-established during healing, providing physiological relevance.

Major concerns:
• The core conclusion of the manuscript, that nuclear IkB plays a role in supressing a fetal phenotype, is based on data from a KO model that, as suggested above, is a selected population. Furthermore, this KO is in both cytoplasmic and nuclear IkB. Independent lines of evidence would strengthen this conclusion. For example, the authors could deplete IkB in WT organoids to

determine whether there is reversion to a fetal-like state and express wild type IkB, or IkB lacking a functional nuclear import signal, in KO organoids to determine whether this reverts them to a differentiated, wild type state.
• The argument that IkB is acting through PRC2 was not that well developed and distracts from the main conclusion of the manuscript.
• On the whole I found the figure legends to be incomplete- lacking information critical for robust data analysis. For example, the number of mice used, the numbers of crypts/mice assessed in image quantification experiments, explanation of data points on graphs etc. some more details are below.

Specific concerns:
Figure 1. D and E, the authors don't give a clear indication of the number of mice (both) and the number of crypts (D) the quantification is based on. F: Overall, less than 50% of LGR5 positive cells showed IkB positivity. The authors suggest it is the EpHB2 population that is positive. Therefore, they should provide quantification for this. H: it would be useful for the authors to definitively prove that this higher band is sumoylated IkB.

Figure 2. D There is no explanation of what was actually quantified. Use of the IkB NES mice is important because it provides the only evidence that the effects observed are solely due to nuclear IkB. This should be in the main figure and experimentation shown to prove that Ikb solely in the nucleus in these tissues.

Figure 3. The number of mice the experiments are based on should be in the figure legends. C is confusing. The analysis was based on 2 WT mice and 3KO mice but there are only 2 points for the KO, EpHB2 low population. Further explanation for this figure is needed. F: the image was very poor-writing for graph legends and axis too small to read. G and H Is it not surprising to see such significant differences in the gene signature of the LGR5 positive population when overall, less than 50% of these cells showed nuclear IkB (Figure 1). Were you able to look at nuclear Ikb in sorted cells.

Figure 4: The most likely explanation for loss of IkB causing changes in gene signature is altered NF-KB activity. Therefore, the data showing conversion to a fetal gene signature is NF-kB independent is important. The manuscript would be improved if all these data were grouped together. I did not find the data in figure 4A completely convincing as there was no quantification and the legend for 4B lacked information and statistics, so analysis was difficult. However, the data in figure S6A-C was more convincing. 4D: how many crypts per animal were quantified?

Figure 5: The data in this figure are convincing and demonstrate clearly that organoids from IkB KO mice have a fetal like gene expression pattern and altered phenotype. But, were the organoids that the micro-array analysis was performed on from one or multiple mice?

Figure 6: The images are not convincing. The EPZ inhibitor does not look like it had a significant effect on the morphology of the organoids. It would be good to show the changes with time that are not depicted. Is there a way to quantify this? For the qPCR it is not clear if the points on the graphs represent technical or biological repeats. Throughout, "3 independent experiments" is used without a clear indication of what this means. Given there are a different number of points per graph, an explanation is required.

Figure 7: I found the explanation of why the effect was IkB dependent and Nf-KB independent confusing. This needs further clarity.

Minor points:

The introduction could spell out the importance of the question being asked and the focus of the study more clearly.

The discussion should bring together the arguments to support the conclusions more robustly- especially the role of PRC2.

Referee #1:

In this study, Marruecos et al investigate the role of IkappaB-alpha in intestinal stem cells. They identify a subset of cells that present nuclear IkappaB-alpha. Using genetic knockouts they associate the phonotype of deficiency with a fetal signature. Finally, they associate this fetal phenotype with a problem in PRC2-function. Overall, this is an interesting study identifying a previously uncharacterized function of IkappaB-alpha, in particular nuclear IkappaB-alpha. The presented data is of very good quality and broadly supports the claims of the authors.
However, **the weakest part of the study is PRC2 connection. Even though, there is association of the genes identified to be occupied by IkappaB-alpha and RNA regulated, there is little mechanistic evidence for the role of PRC2 in this. It is mostly a statistical probability. So, analysis of the order of events, which comes first, PCR2 and then IkappaB, or the other way around, is necessary.**

Answer: We have included new data showing the direct association of nuclear IκBα with PRC2 in the intestinal crypts. In the text in page 8: "by Co-IP experiments we demonstrated the interaction of the PRC2 subunit SUZ12 with SUMOylated IκBα in the nucleus of crypt cells (Figure 4G)." Related with the order of events, the absence of alterations in the analysis of H3K27me3 mark in the developing intestine (embryo E14.5) that are detected at post-natal day 6 (Figure 4I) strongly suggest that PRC2 plays an early IκBα-independent function, and then IκBα restricts or modulates PRC2 activity at particular gene promoters including those regulating ISC maturation and function. We now mention this in the discussion section in page 14. "Since H3K27me3 ChIP from WT and KO embryos at early developmental stage (E14.5) do not show significant differences, we speculated that repression of the fetal ISC phenotype and maturation towards the adult state mediated by IκBα through PRC2 may occur at later stages of intestinal development once villi are formed and differentiated cells start to appear. Thus, PRC2 activity is likely to show two different waves of activity in the intestine, a first general wave that is IκBα independent and a second one that is IκBα dependent and affects specific subsets of genes including those related with ISC maturation from a previous fetal state."

Does IkappaB physically associate with PRC2 components, and is this required for activity? Also controls of PRC2 component levels would be required.
Answer: As mentioned, we have now included a Co-IP experiment showing direct association o SUMOylated IκBα with SUZ12 (Figure 4G). Moreover, we have also included a table showing that mRNA levels of different PRC2 subunits and other related elements are not significantly modified in IκBα deficient intestines. In the text in page 8 we now mention: "IκBα KO displaying a significantly higher number of EZH+ cells per crypt-villus unit (Figure 4E), although we did not observed any significant alteration at the transcriptional level, except for slight increase of Suz12 in KO cells (Figure 4F)."
The mechanisms by which IκBα modulates PRC2 is not addressed in this work, but we previously demonstrated that chromatin binding/dissociation of PRC2 elements to a subset of developmental-related genes is modulated by cytokines in an IκBα-dependent manner. Whether this requires direct binding of IκBα to PRC2 remains to be demonstrated.

The organoid studies are interesting, but some controls are missing. The phenotype is similar to that of APC mutation. Is APC affected, either in level or activity? Similar, is beta-catenin activity altered?
Answer: We have now included a table in new figure Figure EV4F showing comparable mRNA levels of canonical Wnt targets in WT and KO organoids, suggesting that neither beta-catenin nor APC function are altered following IκBα depletion. We mention these results in the text in page 11: "we did not observe any evidence of differentiation but a general decline in organoid growth following Wnt/β-catenin pathway inhibition with XAV939 or the Porcupine inhibitors (Figure EV4). These results are in agreement with absence of Wnt target genes over-activation in IκBα KO organoids as determined by analysis of transcriptomic data (Figure EV4F)."

Finally, how much of this is NF-kappaB-dependent is not clear. It is surprising that no overt activation of NF-kappaB is detected.

Answer: we agree with the reviewer that this is surprising and it was our first hypothesis. For this reason, we are including in the paper several experiments focused on investigating the possible impact of NF-κB in the observed phenotype.

On a minor point, it would be ideal to have single channel images with scale bars on the IF data. It is hard to make out nuclear areas with the images provided.
Answer: we apologize if some images are difficult to visualize. We included the single channel images in EV1A, C and D.

Referee #2:

In this manuscript, the authors demonstrate that phosphorylated IkB is present in the nucleus of intestinal stem cells and that loss of this gene causes these cells to revert to a fetal-like phenotype/gene expression profile. They also demonstrate that nuclear IkB is lost during acute damage of the intestine and reappears in regenerating areas. Based on these data, they conclude that nuclear IkB allows intestinal stem cells to convert from a fetal like state to an adult state during development and that loss of nuclear IkB may play a role in tissue regeneration by allowing cells to revert to a fetal, proliferative state. They provide some evidence that this occurs through altering the PRC2 complex and thus, methylation, although this is secondary to the main conclusion.

I found the manuscript to be of potential interest. It was hard to robustly review, as many details on data acquisition, data analysis and statistics were lacking from both the figure legends and methods.
Answer: We apologize for the omission. We have now included in the text and figure legends all the information related with the number of replicates and mice, and intestinal crypts counted.

Below I have listed (based on the data presented) what I believe to be the main strengths and weaknesses.

Strengths: The authors reveal a novel role for IkB in the intestine that builds on recent data demonstrating that tissue damage causes intestinal stem cells to revert to a fetal state. Fuller understanding of this reversion may help develop strategies to accelerate wound healing in this and other tissues. **Therefore, the finding in the manuscript, that IkB may play a role, could be of extreme interest to the field. Although the conclusions are mostly based on a knockout mouse model, they do present data demonstrating loss of nuclear IkB in damaged tissue, that is re-established during healing, providing physiological relevance.**
Answer: We thank the reviewer for these comments.

Major concerns:
• The core conclusion of the manuscript, that nuclear IkB plays a role in suppressing a fetal phenotype, is based on data from a KO model that, as suggested above, is a selected population. Furthermore, this KO is in both cytoplasmic and nuclear IkB. Independent lines of evidence would strengthen this conclusion. **For example, the authors could deplete IkB in WT organoids to determine whether there is reversion to a fetal-like state and express wild type IkB, or IkB lacking a functional nuclear import signal, in KO organoids to determine whether this reverts them to a differentiated, wild type state.**
Answer: We have now generated IκBα KO organoids by CRISPR-Cas9 and found that IκBα depletion does not revert the differentiated phenotype of already formed organoids into a fetal phenotype, under standard culture conditions. These results suggest that maturation of fetal ISC into adult ISC that depends on IκBα is defined during embryonic development (most likely after E14.5 and before P6 in mice based on H3K27me3 ChIP-seq data) and does not require persistent IκBα activity, at least under homeostatic conditions, which seems to be different under damage conditions. In the discussion we have included this concept: "This requirement seem to be different in non-homeostatic in vivo conditions, since IκBα loss in the damage tissue is associated with a partial conversion of cells into a transient fetal-like phenotype (revealed by increased expression of the ISC genes Olfm4 and Cd44)." In the results section in page 10, we have included this data: "We next knocked out IκBα by CRISPR-Cas9 in already formed WT organoids (Figure 5F). CRISPR-Cas9 KO of IκBα in WT intestinal organoids did not significantly affect their differentiated

morphology (Figure 5G), suggesting that regulation of ISC maturation by IκBα is exerted during development and is not continuously required to maintain adult ISCs under standard growing conditions."
Reconstituting KO with WT or IκBα mutant lacking nuclear import signal is an interesting idea, however nuclear-cytoplasmic shuttling of IκBα is required not only for its PRC2 related function but for proper inhibition of NF-KB factors (Arenzana-seisdedos et al. J. Cell Sci. 1997). Thus, we are currently investigating the upstream elements that impose chromatin association of IκBα to perform the suggested experiments in the most informative conditions.

• The argument that IkB is acting through PRC2 was not that well developed and distracts from the main conclusion of the manuscript.
Answer: To our view, the likely possibility that IκBα is acting through PRC2 to regulate ISC-related gene expression is relevant enough to be included in the paper. To re-enforce the concept that IκBα acts though PRC2 in the intestinal crypt we have included a Co-IP experiment showing physical association between nuclear SUMOylated IκBα from intestinal crypt cell lysates and SUZ12 (new Figure 4G). Further investigating when and how ISC genes such as Ascl2 or Lgr5 are activated during development and are induced by cytokines in an IκBα-dependent manner is, to our view, out of the scope of this work, and it is the objective of further studies that we will carry out by single promoter ChIP analysis.

On the whole I found the figure legends to be incomplete- lacking information critical for robust data analysis. For example, the number of mice used, the numbers of crypts/mice assessed in image quantification experiments, explanation of data points on graphs etc.
Answer: We have revised the manuscript to improve clarity and include relevant information that was lacking.

Specific concerns:

Figure 1. D and E, the authors don't give a clear indication of the number of mice (both) and the number of crypts (D) the quantification is based on.
Answer: We have included this information in the Figure legends.

F: Overall, less than 50% of LGR5 positive cells showed IkB positivity. The authors suggest it is the EpHB2 population that is positive. Therefore, they should provide quantification for this.
Answer: We have included this information in revised Figure 1F.

H: it would be useful for the authors to definitively prove that this higher band is sumoylated IkB.
Answer: We have added a new figure showing that nuclear IκBα obtained from intestinal crypt cells is precipitated with the anti-SUMO2/3 antibody (new Figure 1I).

Figure 2. D There is no explanation of what was actually quantified. Use of the IkB NES mice is important because it provides the only evidence that the effects observed are solely due to nuclear IkB. This should be in the main figure and experimentation shown to prove that Ikb solely in the nucleus in these tissues.
Answer: We have included results from IκBα NES mice in the main figures 2F and 2G. Moreover, we have performed IHC analysis of P-IκBα in the intestinal sections of these animals and detected increased levels of nuclear P-IκBα that was still restricted to the crypt areas (Figure 2F).

Figure 3. The number of mice the experiments are based on should be in the figure legends. C is confusing. The analysis was based on 2 WT mice and 3KO mice but there are only 2 points for the KO, EpHB2 low population. Further explanation for this figure is needed.
*Answer: We included the information in Figure legend: "*3 mice per genotype were initially processed although 1 EphB2^{neg} KO replicate was excluded from the analysis due to insufficient number of reads in the RNA seq."

F: the image was very poor-writing for graph legends and axis too small to read. G and H Is it not surprising to see such significant differences in the gene signature of the LGR5 positive

population when overall, less than 50% of these cells showed nuclear IkB (Figure 1). Were you able to look at nuclear Ikb in sorted cells.
Answer: We have modified the figures to facilitate reading, and included the IF analysis of IκBα in sorted EphB2 high/negative cells (new figure 3C). About G and H (now H and I), although it is true that only half of LGR5+ cells are P-IκBα positive (based on IHC analysis), most of them contain nuclear IκBα when detected with the non phospho-specific antibody (Figure 1B). Most importantly, we do not think that phosphorylated IκBα is restricted to a subset of the Lgr5+ cells (that constitute a very homogeneous population) but instead nuclear IκBα is cycling between the non-phosphorylated and phosphorylated forms (in response to specific stimuli not yet defined) leading to waves of gene activation/repression, but this is something that we are still investigating. In the discussion section we mention this possibility.

Figure 4: The most likely explanation for loss of IkB causing changes in gene signature is altered NF-KB activity. Therefore, the data showing conversion to a fetal gene signature is NF-kB independent is important. The manuscript would be improved if all these data were grouped together.
Answer: We agree with the reviewer that altered NF-κB activity in the IκBα KO mice is the easiest explanation for the observed phenotype, and this was our first idea too. For this reason, we have now moved all data from NF-κB KO organoids to the main figures 6A and 6B. There is also relevant data in Figure 7D showing that Cd44 and Olfm4 activation by TNFα in the organoids is IκBα but NF-κB independent, since KO organoids are no able to induce them but are still able to induce known NF-κB target genes such as Cxcl10 or A20.

I did not find the data in figure 4A completely convincing as there was no quantification and the legend for 4B lacked information and statistics, so analysis was difficult. However, the data in figure S6A-C was more convincing.
Answer: We have repeated the experiments shown in 4A several times and using different antibodies against c-Rel and p65, and we have never detected nuclear NF-κB proteins in the WT or KO intestinal crypts. This is why we have not included any quantification. About Figure 4B, we have included the required information in the legend: "Number of peaks from p65 ChIP-sequencing analysis associated with different genomic localizations was obtained merging two biological replicates per condition (n=2 P6 WT and n=2 P6 IκBα KO intestinal crypt cells)." because of the experimental approach, there is no possible statistics test to be applied, however, values were practically identical between WT and KO, as it is also shown in the p65 ChIP examples in Figure EV3A.

4D: how many crypts per animal were quantified?
Answer: We have included this information in the figure legend.

Figure 5: The data in this figure are convincing and demonstrate clearly that organoids from IkB KO mice have a fetal like gene expression pattern and altered phenotype. But, were the organoids that the micro-array analysis was performed on from one or multiple mice?
Answer: we have included this information in the figure legend: "5 technical replicates of a minimum of two organoids per genotype were analyzed".

Figure 6: The images are not convincing. The EPZ inhibitor does not look like it had a significant effect on the morphology of the organoids. It would be good to show the changes with time that are not depicted. Is there a way to quantify this?
Answer: We have included new pictures showing changes overtime and an approximate quantification of the morphological changes observed (new Figure 6C).

For the qPCR it is not clear if the points on the graphs represent technical or biological repeats. Throughout, "3 independent experiments" is used without a clear indication of what this means. Given there are a different number of points per graph, an explanation is required.
Answer: points include both technical replicates and several (at least 3 independent experiments). We are now including additional experiments to show comparable number of values to all graphs.

Figure 7: I found the explanation of why the effect was IkB dependent and Nf-KB independent confusing. This needs further clarity.

Answer: We have modified the text to improve clarity. We now say in page 12: Olfm4 and Cd44 transcription was induced by the pro-inflammatory cytokine TNFα in intestinal organoids, which was IκBα dependent (they are not induced in IκBα KO organoids at any time point of TNFα treatment) but NF-κB independent (since canonical NF-κB targets such as Cxcl10 and A20 are consistently induced by TNFα in KO organoids) (Figure 7D).

Minor points:

The introduction could spell out the importance of the question being asked and the focus of the study more clearly.

Answer: We have now included a sentence trying to focus on the importance of the question asked: "The possibility that IκBα, in an NF-κB-dependent or independent manner, participates of ISC regulation under physiologic or pathologic situations have not been specifically addressed."

The discussion should bring together the arguments to support the conclusions more robustly- especially the role of PRC2.

Answer: We have included two paragraphs in the discussion section highlighting the connection between nuclear IκBα and PRC2. Specifically, we now mention: "we were able to directly demonstrate the physical association between nuclear SUMOylated IκBα and the PRC2 subunit SUZ12 in IP experiments from intestinal crypt cell lysates, further indicating that intestinal IκBα modulates PRC2 function" and also "Thus, PRC2 activity is likely to show two different waves of activity in the intestine, a first general wave that is IκBα independent and a second one that is IκBα dependent and affects specific subsets of genes including those related with ISC maturation from a previous fetal state" and at the end of the discussion "Our hypothesis, awaiting further substantiation, is that IκBα acts as a molecular switch for stimulus-mediated activation of a subset of ISC genes, likely through modulation of PRC2 activity."

2nd Editorial Decision 26 February 2020

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, both referees now support the publication of your study in EMBO reports. However, referee #1 has two remaining points we ask you to address in a final revised version. In particular, please add the requested input controls.

Further, I have these final editorial requests:

- Please name the summary 'abstract'.

- Please provide one section for the material and methods termed 'Material and Methods'.

- There are tables in the methods section that need naming. I suggest moving these in an Appendix file. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Table Sx etc. throughout the text, and also label the tables according to this nomenclature in the Appendix. Please provide the Appendix as one single pdf file.

- Please change the name of the paragraph 'Declaration of interests' into 'Conflict of interest statement'.

- Please format the references according to our journal style. See:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- As already indicated in my first decision letter: Please refrain from writing the size on or near the scale bars. Please provide the size information only in the respective figure legends.

- For the bar diagram in figure 7E you indicated for the KO n=2. Does that mean that for the KO you used 2 mice to analyse ulcerations (and 3 wt). How many images per mouse were quantified (technical replicates)? Presently, the information here is too scarce. However, if indeed only two

images were analysed, error bars and statistical testing do not make much sense, and should be removed. Please address this.

- Please add a scale bar to the magnification box in Fig. 1A, or define the size of the box in the legend.

- Authors Monica Gonzalez-Farre and Sara Arce-Gallego are missing from the author contributions. Please check.

- Please enter the funding information also into our submission system. Please check that in the online form and the manuscript the funding information is the same and complete.

- Finally, 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. I think you have already addressed these. But please re-check and provide your final manuscript file with track changes, in order that we can see the modifications done.

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 authors addressed all of my concerns and provide evidence for the less robust areas of their original submission. The new Co-IP data however, is missing input controls which are very important for the figure.

I still have reservations regarding the statement of this being completely NF-kappaB independent, since this can not really be proven in their system. The authors have also to rephrased this in my opinion.

Referee #2:

I am happy that the authors have addressed all my original concerns. The figure legends now contain the relevant information and I am convinced that the data are strong and well controlled.

I am glad the authors attempted the KO experiment in organoids. It is interesting that once the adult signature has been established, it cannot be reverted. it will now be interesting to understand how this happens in damaged tissue.

In my opinion, the manuscript now presents clear evidence that nuclear IkB prevents a fetal like state in intestinal stem cells, at least in part through modulation of the PRC2 complex. I believe this will be of interest to a number of fields.

2nd Revision - authors' response 28 February 2020

The authors performed all minor editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Lluis Espinosa
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49708V1

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.

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Every experiment was repeated at least 3 independent times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was determined by the number of pups available in each experiment. All the experiments were repeated until a minimum of 3 mice in each condition were analysed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples with technical problems such as histological section quality 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.	All the analyzed mice had the same age (2 month old mice for general analysis and P4-P6 for IkBa KO analysis) to avoid developmental differences.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were crossed with inbred colonies and mice from the same litter were used for controls in each experiment. Enough mice were used to reach a minimum of 3 replicates per experiment.
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.	Quantification of cells and immunofluorescence were performed by at least two blinded people.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done for animal studies
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis has been performed using GraphPad Prism6 software (GraphPad) and p<0.05 is considered significant. Two-sided Student's t-test was used to compare differences between two groups.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not use any test for normal distribution

Is there an estimate of variation within each group of data?	There is no estimate of variation within each group of data
Is the variance similar between the groups that are being statistically compared?	The variance is similar between compared groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog number has been provided
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in this study

* 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.	WT mice C57BL/6J were purchased from The Jackson Laboratories. IκBα knock out (KO) (B6;129S4-Nfkbiatm1Bal/J) mice and Lgr5GFP-CreERT (B6.129P2-Lgr5tm1(cre/ERT2)Cle/J) mice have the same genetic C57BL/6J background and were obtained from The Jackson Laboratories and IκBαNES were previously described (Wuerzberger-Davis et al., 2011). Compound IκBβ;IκBε KO mice were generously donated by Dr. Alexander Hoffmann (University of California, LA).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	In all procedures, animals were kept under pathogen-free conditions, and animal work was conducted according to the guidelines from the Animal Care Committee at the Generalitat de Catalunya. The Committee for Animal Experimentation at the Institute of Biomedical Research of Bellvitge (Barcelona) approved these studies.
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.	Ww comply all guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	Accession codes for datasets have been provided.
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).	Data has been deposited at GEO
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).	NA
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.	NA

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.	NA