EMBO
reports

Sirt1 protects from K-Ras-driven lung carcinogenesis

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1st Editorial Decision 5 January 2017

Thank you for the transfer of your research manuscript to EMBO reports. I now went through the referee reports from The EMBO Journal.

All referees acknowledge the potential interest of the findings. Nevertheless, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here.

As EMBO reports emphasizes novel functional over detailed mechanistic insight, we will not require to address the points regarding more refined mechanistic details and cell autonomy (if you have data addressing this, we would of course welcome their inclusion in a revised version). However, we think all other points of referees #2 and #3 need to be addressed during a revision.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in the revised manuscript (as detailed above) and in a complete 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Please refer to our guidelines for preparing your revised 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. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

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

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- 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)

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 about 400 pixels) that can be used as part of a visual synopsis on our website.

Please note that we now mandate that all corresponding authors list an ORCID digital identifier!

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:

This paper demonstrates a tumor suppressive role of SIRT1 in the development of KRas-driven lung adenocarcinomas in mice and humans. The paper has no mechanistic insight and does not distinguish between cell-autonomous and nonautnomous functions of SIRT1 suppressing lung cancer. In the original paper in PNAS by Pfluger et al. 2008, the SIRT1 TG is a global TG. It would

have been important to induce expression only in the cancer cells to examine whether the effects are cell autonomous. This is a critical experiment lacking. Furthermore, the RNA seq. is just data overload without any mechanistic insight. Again the Pfluger paper demonstrated that SIRT1TG decreased IL-6, a known cytokine that promotes lung cancer. Maybe SIRT1Tg is modulating inflammation. Overall the paper has not provided any mechanistic insight into lung cancer or provided a new potential therapeutic target.

Referee #2:

In this manuscript, Herranz and colleagues demonstrate a tumor suppressive effect of SIRT1 in KRas-driven lung carcinogenesis. SIRT1 has been reported to have a complex role in carcinogenesis, working sometimes as a tumor suppressor and sometimes as an oncogene, in a tissue and context-dependent manner. The role of SIRT1 in KRas-driven lung carcinogenesis has not been explored, and as such this study has the potential to shed some new light into this particular role for this deacetylase. As described below in detail, although the in vivo experiments where SIRT1 overexpression in transgenic mice delay KRas-driven lung carcinogenesis are quite intriguing, mechanistic details on the link between SIRT1 and KRas are poorly investigated. Most of the data shown in this manuscript is rather descriptive, and some experiments, as presented, seems preliminary and do not support the authors' conclusions. As it stands, the manuscript does not warrant publication in EMBO J.

Major concerns:

- In Figure 1A, 1B, 2A, and 2B, the authors use primary MEFs from either WT or SIRT1-Tg mice. Since primary MEFs are known to be highly heterogeneous and to undergo senescence without further immortalization, it is difficult to assess whether decreased expression of SIRT1 over days in cell culture is truly due to KRas as claimed by the authors. Senescence could be a main driver of SIRT1 expression. To tackle this problem, the authors need to immortalize primary MEFs in order to bypass senescence (such as p53 knockdown) and show at least three independent MEF lines to have consistency of the data.
- In Figure 1C and 1D, the authors use KRasG12V KI MEFs over WT MEFs with or without SIRT1 overexpression. Here, since KRas activation itself can easily induce oncogene-induced senescence (OIC), the authors should perform β-gal staining to verify whether these cells do not undergo massive senescence. In addition, given the effect of passage on SIRT1 levels (as shown in 1A), it is difficult to assess whether infection with KRas-mutant caused the reduction in SIRT1 levels, or rather it is an effect of passaging the cells. The authors should infect the cells with adeno-control virus, and collect at the same time-point as the Adeno-Cre infected cells, to compare Sirt1 levels. In addition, it seems WT Ras also has an effect on SIRT1. Given the known variability observed in MEFs, the authors should include more independent replicates in this experiment to conclusively demonstrate that mutant KRas, specifically, influences SIRT1 levels.
- In this context, it remains unclear what is the proposed mechanism of SIRT1regulation by mutant KRas. Given that mRNA levels are not affected, we have to assume that SIRT1 is regulated at the level of protein stability. How so? Is it through active degradation? Does it involves a phosphorylation event? (previous studies have demonstrated phosphorylation regulation of SIRT1). Without some mechanistic insights in this regard, the hypothesis of the authors remains highly speculative.
- In Figure 2A and 2B, although both inhibitor treatments worked well, changes in SIRT1 expression in the context of the inhibitors looks minimal and highly variable. Here again, since this effect may be due to senescence of primary MEFs, they also need to do the same experiment in KRasG12V KI MEFs to confirm this is truly a KRas-dependent effect, and add more replicates.
- In Figure 2C and 2D, the authors should first check SIRT1 protein levels and KRas activation across all the cell lines side by side to see if there is a correlation between KRas activation state and SIRT1 protein expression. As shown, the figure seems random. For instance, it will be reassuring if the four cell lines that showed increased SIRT1 levels following inhibitor treatment have KRas activating mutation(s), which would explain why they are sensitive to the inhibitors.
- In Figure 4, the authors utilized a pulse-chase system with tamoxifen-containing diet. We could not understand what was the benefit to use this pulse-chase system since upon Cre recombinase activation by tamoxifen, KRasG12V+ and KFP+ cells are permanently generated. In addition, the authors should provide a scientific rationale why the number of KFP+ cells is decreased after 2 weeks of chase (due to senescence or clearance by immune system, etc). Since induction of KFP

expression is permanent after Cre recombinase activation, we have to speculate that for some unclear reason these cells are dying (or more likely senescing), in which case preparing RNA from the few remaining cells (many of which may be dying) adds noise to the experiment. The authors should compare the SIRT1-TG and WT KFP expressing cells following few weeks of Tamoxifen treatment, without the chase.
• In Figure 4D, the authors summarize the differentially expressed genes from the pneumocytes in SIRT1 WT or overexpressing Tg animals. As presented, the list of genes/pathways is descriptive. In order to gain mechanistic insights, at least some follow-up experiments should be done to address which pathway(s) plays driving roles downstream of SIRT1 deficiency.
• In the discussion, the authors highlight metabolic networks in the context of SIRT1. However, such discussion seems irrelevant in a manuscript where the authors did not provide any relevant data related to metabolism.

Minor concerns:
• In Figure 1B, mRNA expression of SIRT1 rebounds at day 7 in culture. This seems like an intriguing observation that worth some discussion.
• In Figure 2C, some of the western blots for pERK, pAKT, and pFOXO are of poor quality. The authors should clearly show this by redoing western blots to prove if the inhibitor treatment worked in the conditions they used.
• In Figure 3, the authors injected tamoxifen to induce Cre recombinase activity for KRasG12V KI. Please specify which Cre-ER system was used in this experiment. Some Cre-ER lines are known to be leaky, and therefore describing the line used (or confirming deletion only upon treatment) will be reassuring.
• In Figure 4, the authors should check if most of the KFP+ cells have KRasG12V activation at the same time. Different lox-stop-lox cassettes have different recombination efficiency in the presence of Cre recombinase. The authors cannot assume that most of the KFP+ cells indeed have activated KRas.

Referee #3:

SIRT1 is s member of the Sirtuin family of NAD+-dependent deacetylases involved in stress response signaling. The role of Sirt1 in cancer is complex and seems to depend on the functional context as it has been shown upregulated or downregulated in a wide range of cancers. In the current manuscript by Herranz et al., the authors study a direct link between the SIRT1 and KRAS in lung cancer. The authors observe a decrease in SIRT1 protein levels upon induction of KRAS mutant G12V and link it to MAPK pathway signaling. Moreover, they demonstrate that the formation of lung adenocarcinomas in mice produced by KRAS-G12V expression is delayed by SIRT1 increased levels (sirt1-tg mice). The authors show that higher levels of SIRT1 in these animals correlate with an increased lifespan in 24% and a lower rate of carcinomas. Interestingly, SIRT1 did not have any effect on the formation of lung adenomas, a previous step to formation of carcinomas, or in tumor growth once had appeared. Confirming their claims, the authors also analyze a set of NSCLCs human tumors and correlate SIRT1 increased levels with longer overall survival. To study the changes associated to SIRT1 expression, the authors perform RNAseq in isolated pneumocytes from KRASG12V/SIRT1Tg animals and identify several cancer-related genes downregulated by SIRT1 induction.
Overall, this is a very interesting set of findings. Although the protective role of SIRT1 in cancer has been suggested before, the role of SIRT1 in lung carcinogenesis had not been well defined. Previous studies already described a functional interplay between SIRT1 and KRAS signaling (Cheng et al, 2015), including KRAS deacetylation by SIRT1, in established non-small cancer cells. However, these studies were entirely based on lung cancer cell lines. Interestingly, in this work the authors follow the in vivo development of lung tumors and observe that the protective effect of SIRT1 is relevant mainly in the first stages of tumorigenesis. This is a very relevant issue, as the vast majority of studies on the role of SIRT1 in tumorigenesis have been performed in established tumors or cell lines derived from them. This work may help reconcile the observation that SIRT1 is found both downregulated and upregulated in cancer as suggests that SIRT1 prevents tumor formation but may be required later for tumor development. However, there are several major issues that limit considerably the relevance of the work:
1) I have problems interpreting the data in Figures 1c and 2. The observed effect of KRAS on SIRT1

levels is mild and the authors should be very careful about drawing conclusions from these experiments. In Figure 1c, the only clear conclusion is that the profile of SIRT1 levels between KRAS WT or G12V is different at day 4. This could be relevant for the claims of the authors as it supports a differential effect of both KRAS forms towards SIRT1 in early stages of KRAS induction (between 0-4 days). Unfortunately, it is based on a single point (4d) and should be developed further to make a convincing claim. One possibility would be to also show the levels of SIRT1 between day 1-4. A second possibility would be to demonstrate this effect through another approach. If the authors claim that KRAS mutant inhibits SIRT1 more than WT at the protein level, the half life of SIRT1 protein should be different. To test that, the authors could study SIRT1 stability under these conditions around 4 days by Cycloheximide (CHX) treatments.
2) In the same line, in Figure 2 the effect of MEKi or PI3Ki treatment on SIRT1 levels is quite different between replicates. This makes difficult to draw any clear conclusion, particularly in fig 2B quantification, as the levels of SIRT1 between DMSO, MEKi or PI3Ki do not seem to be statistically significant at day 4. The authors should provide more duplicates to improve statistics. Additionally, an alternative possibility could also be to perform CHX studies with these treatments. If included, this would strengthen considerably the claims of the manuscript.
3) In Figure 1c experiments, is there any difference in the proliferation rate of KRAS-KI between WT and Tg SIRT1? Additionally, does either inhibition (e.g Ex-527) or downregulation of SIRT1 alter KRAS-KI induced proliferation? Any data in this direction would strengthen the antagonism between KRAS- G12V and SIRT1.
4) How do the authors explain that of all 8 cell lines tested in Fig. 2c-d, only 4 show SIRT1 upregulation? In fact, only in 2 of these lines, the upregulation of SIRT1 shows a two-fold increase. These issues should be discussed if the authors claim a general effect of SIRT1 in lung cancer. Moreover, the lack of statistical analysis in Fig. 2D is also a concern.
5) As stated by the authors, several studies suggested that SIRT1 expression actually associates with poor prognosis (Noh et al., 2013; Grbesa et al, 2015; Lin and Peng, 2016). Tha authors explain the discrepancy because these studies seemed to analyze mainly squamous cell carcinomas rather than adenocarcinomas. However, at least another study (Li et al, Onco Targets Ther, 2015) makes that claim directly on adenocarcinomas. These studies contradict the results in figure 3F. The authors should discuss these differences.

1st Revision - authors' response — 4 May 2018

EMBOR-2016-43879V2
Response to Reviewer's comments.

[AUTHORS] We thank the reviewers for their time and careful evaluation of our manuscript. Their comments have helped us to improve the paper. We have addressed all their comments, as explained below, including the addition of new data and modifications to the text. We trust that the reviewers will now find the manuscript appropriate for publication in *EMBO Reports.*

We also thank the reviewers for their encouraging comments:

Reviewer #2: "*The role of SIRT1 in K-Ras-driven lung carcinogenesis has not been explored, and as such this study has the potential to shed some new light into this particular role for this deacetylase*".

Reviewer #3: "*Overall, this is a very interesting set of findings… This is a very relevant issue… This work may help reconcile the observation that SIRT1 is found both downregulated and upregulated in cancer.*"

Reviewer #2:

In this manuscript, Herranz and colleagues demonstrate a tumor suppressive effect of SIRT1 in K-Ras-driven lung carcinogenesis. SIRT1 has been reported to have a complex role in carcinogenesis, working sometimes as a tumor suppressor and sometimes as an oncogene, in a tissue and context-dependent manner. The role of SIRT1 in K-Ras-driven lung carcinogenesis has not been explored,

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and as such this study has the potential to shed some new light into this particular role for this deacetylase. As described below in detail, although the in vivo experiments where SIRT1 overexpression in transgenic mice delay K-Ras-driven lung carcinogenesis are quite intriguing, mechanistic details on the link between SIRT1 and K-Ras are poorly investigated. Most of the data shown in this manuscript is rather descriptive, and some experiments, as presented, seems preliminary and do not support the authors' conclusions. As it stands, the manuscript does not warrant publication in EMBO J.

Major concerns:

1.- In Figure 1A, 1B, 2A, and 2B, the authors use primary MEFs from either WT or SIRT1-Tg mice. Since primary MEFs are known to be highly heterogeneous and to undergo senescence without further immortalization, it is difficult to assess whether decreased expression of SIRT1 over days in cell culture is truly due to K-Ras as claimed by the authors. Senescence could be a main driver of SIRT1 expression. To tackle this problem, the authors need to immortalize primary MEFs in order to bypass senescence (such as p53 knockdown) and show at least three independent MEF lines to have consistency of the data.
[AUTHORS] We heartfully thank the reviewer for his/her comment, and we completely agree with him that a main driver of the observed decrease in Sirt1 expression can be the oncogenic signaling during cell culture, that leads to the senescence response. Actually, when we follow this reviewer's suggestion and measure Sirt1 expression in immortalized MEFs, we cannot detect any effect in Sirt1 protein expression (New Figure S1B). We have commented about this finding in the text: "*Sirt1 decrease only takes place when the cellular response to culture stress is intact (as in primary MEFs), but not when this response is lost (as in immortalized MEFs)*".

2.- In Figure 1C and 1D, the authors use K-RasG12V KI MEFs over WT MEFs with or without SIRT1 overexpression. Here, since K-Ras activation itself can easily induce oncogene-induced senescence (OIC), the authors should perform β-gal staining to verify whether these cells do not undergo massive senescence.
[AUTHORS] We fully agree with the importance of detecting oncogene-induced senescence in our MEFs, specially after our finding that immortalized MEFs do not show the decrease in Sirt1 protein levels with time of passage. In new Figure S1D (previous Figure S1B) we show that K-Ras-KI MEFs do not arrest in their proliferation, but instead they continue growing at a slightly quicker pace, indicating that no overt senescence is taking place. This finding was already shown when the K-Ras-KI model was first described[1]. In addition, since the K-Ras-KI model includes a IRES-cassette linking the ORF of K-Ras with a β-galactosidase reporter gene, we cannot detect a senescence-specific staining with these cells. Indeed, we have used this reporter to monitor K-Ras activation (see Figure S1C), but it does not indicate any onset of senescence.

In addition, given the effect of passage on SIRT1 levels (as shown in 1A), it is difficult to assess whether infection with K-Ras-mutant caused the reduction in SIRT1 levels, or rather it is an effect of passaging the cells. The authors should infect the cells with adeno-control virus, and collect at the same time-point as the Adeno-Cre infected cells, to compare Sirt1 levels.
In addition, it seems WT Ras also has an effect on SIRT1. Given the known variability observed in MEFs, the authors should include more independent replicates in this experiment to conclusively demonstrate that mutant K-Ras, specifically, influences SIRT1 levels.
[AUTHORS]: We had used the Adeno-Cre virus to infect K-Ras-WT MEFs as our control, because Cre itself can promote DNA damage and induce senescence[2]. As shown in New Figure 1B and 1C, after infection with Adeno-Cre virus, Sirt1 levels were more stable in K-Ras-WT MEFs than in K-Ras-KI MEFs.

Following the suggestion of this reviewer, we have infected four new clones of our K-Ras-KI MEFs with Adeno-Cre or with Adeno-GFP as controls, and have measured Sirt1 protein stability. As shown in New Figure 1D, we could observe a significant decrease in Sirt1 protein stability when infected with Adeno-Cre, compared with Adeno-GFP-infected cells.

3.- In this context, it remains unclear what is the proposed mechanism of SIRT1 regulation by mutant K-Ras. Given that mRNA levels are not affected, we have to assume that SIRT1 is regulated at the level of protein stability. How so? Is it through active degradation? Does it involves a phosphorylation event? (previous studies have demonstrated phosphorylation regulation of SIRT1).

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Without some mechanistic insights in this regard, the hypothesis of the authors remains highly speculative.

[AUTHORS] Following this reviewer's suggestion we have studied two possible mechanisms for the regulation of Sirt1: transcriptional regulation and protein stability. For the first analysis, we have obtained the RNA from K-Ras-KI MEFs infected with Adeno-GFP or Adeno-Cre and analyzed Sirt1 transcription levels. Interestingly, we observed decreased transcription of *Sirt1* only the Sirt1-Tg MEFs, while Sirt1-WT MEFs did not change *Sirt1* transcription. This result fits with our prior data in New Figure S1A, obtained with Sirt1-WT and Sirt1-Tg MEFs with days in culture. We have included these data in New Figure S1E and commented about them in the text: "*Interestingly, this reduction in Sirt1 protein was accompanied by a decrease in Sirt1 mRNA only in Sirt1-Tg MEFs, indicating a stronger pressure by K-Ras-activation towards Sirt1 decrease specifically on Sirt1-overexpressing MEFs. Interestingly, Sirt1 mRNA levels rebound slightly after 7 days in culture (Figure 1B), which could indicate a compensatory mechanism for the decreased Sirt1 protein levels and stability observed*".

For the second approach, we have studied the altered stability of the protein Sirt1 as a possible mechanism by which K-Ras induces the global decrease in Sirt1 protein levels. As indicated in New Figure 1D, infection of K-Ras-KI MEFs with Adeno-Cre resulted in reduced protein stability when compared with K-Ras-KI MEFs infected with Adeno-GFP control virus.

We therefore think that time in culture induces a global decrease in Sirt1 levels by two different mechanisms: when Sirt1 is overexpressed, as in Sirt1-Tg MEFs, oncogenic signaling induces a decrease in *Sirt1* transcription, that does not take place when *Sirt1* is expressed at normal levels, as in Sirt1-WT MEFs. In turn, protein levels of Sirt1 are downregulated in both Sirt1-WT and Sirt1-Tg MEFs with time in culture, and this downregulation is due to shorter protein stability, as shown in New Figure 1D.

4.- In Figure 2A and 2B, although both inhibitor treatments worked well, changes in SIRT1 expression in the context of the inhibitors looks minimal and highly variable. Here again, since this effect may be due to senescence of primary MEFs, they also need to do the same experiment in K-RasG12V KI MEFs to confirm this is truly a K-Ras-dependent effect, and add more replicates.
[AUTHORS] As suggested by this reviewer, we have tested Sirt1 protein levels in K-RasG12V-KI MEFs infected with Adeno-GFP or Adeno-Cre. In particular, we tested Sirt1 protein stability after infection, and observed a decrease in Sirt1 stability when K-RasG12V was activated (New Figure 1D). We also observed that this decreased stability in Sirt1 protein was recovered by a MEKi treatment in WT MEFs in culture (New Figure 2B and 2C) and in K-RasG12V-KI MEFs infected with Adeno-Cre (New Figure 2D). After all these evidences, we consider that our argument of decreased Sirt1 protein stability by K-Ras activation has been strongly reinforced, thanks to the suggestion of this reviewer.

5.- In Figure 2C and 2D, the authors should first check SIRT1 protein levels and K-Ras activation across all the cell lines side by side to see if there is a correlation between K-Ras activation state and SIRT1 protein expression. As shown, the figure seems random. For instance, it will be reassuring if the four cell lines that showed increased SIRT1 levels following inhibitor treatment have K-Ras activating mutation(s), which would explain why they are sensitive to the inhibitors.
[AUTHORS] We present K-Ras status in all checked cell lines (Table S1). As shown, Sirt1 levels respond to MEK and/or PI3K inhibition only in lung adenocarcinoma cell lines, and not in cell lines from small cell carcinomas (H841) and large cell carcinoma (H661), suggesting that this response of Sirt1 is specific to adenocarcinomas. Moreover, there are two lung adenocarcinoma cell lines that do not respond to MEKi/PI3Ki: H358 and H23. Interestingly, these cell lines harbor the same K-Ras mutation: G12C, while the other responding cell lines have either WT K-Ras (Calu3) or different mutations of K-Ras (G12S for A549 and G12V for H441). This is interesting, since different mutations in K-Ras have been associated to different prognostic and molecular features. We have included a comment on this issue in the text.

6.- In Figure 4, the authors utilized a pulse-chase system with tamoxifen-containing diet. We could not understand what was the benefit to use this pulse-chase system since upon Cre recombinase activation by tamoxifen, K-RasG12V+ and KFP+ cells are permanently generated. In addition, the authors should provide a scientific rationale why the number of KFP+ cells is decreased after 2 weeks of chase (due to senescence or clearance by immune system, etc). Since induction of KFP expression is permanent after Cre recombinase activation, we have to speculate that for some

unclear reason these cells are dying (or more likely senescing), in which case preparing RNA from the few remaining cells (many of which may be dying) adds noise to the experiment.
The authors should compare the SIRT1-TG and WT KFP expressing cells following few weeks of Tamoxifen treatment, without the chase.
[AUTHORS] Lung airway cells are replaced relatively quickly, taking from a few days in the bronchi to 4-6 weeks in the alveoli [3]. We include an assessment of the K-Ras-KI cells remaining after the 2 weeks of chase in New Figures 4B and 4C. Some of these remaining cells will stay to form the lung tumors. With this rationale, we waited for most of the K-Ras-KI cells to be eliminated, and isolated only the few remaining cells that will give rise to the tumor.
With this in mind, we think that the reviewer's suggestion has addressed an important issue: in the pulse-chase experiment we are only selecting for the cells which have activated K-Ras and reacted to this activation, that is: the RNAseq signature that we are getting here is the reaction to the acute K-Ras-KI activation. However, if we isolate K-Ras-KI-activated cells without any chase phase, we can obtain an RNAseq signature of the acute activation phase. We consider that both approaches are complementary and can yield a valuable information about the mechanism of Sirt1-mediated protection. Therefore, we have followed the reviewer's advice and performed the RNAseq on pneumocytes isolated after a 4 weeks-tamoxifen treatment without any chase phase, that we have included in New Figure 4. We hope these new data and analysis will be of interest for the reviewer.

7.- In Figure 4D, the authors summarize the differentially expressed genes from the pneumocytes in SIRT1 WT or overexpressing Tg animals. As presented, the list of genes/pathways is descriptive. In order to gain mechanistic insights, at least some follow-up experiments should be done to address which pathway(s) plays driving roles downstream of SIRT1 deficiency.
[AUTHORS] Following this reviewer's suggestion, we have performed a thorough analysis of the differentially expressed genes from the two RNAseq experiments. We have carefully assigned them with oncogenic/tumor suppressive functions after a deep literature searching, and have generated lists of genes whose dysregulation can explain the anti-tumoral phenotype of Sirt1 overexpression (New Figure 4D and 4E). We have also compared the differential levels of expression of oncogenes and tumor suppressors in both experiments. Interestingly, we observed that in the pulse experiment, Sirt1-overexpressing pneumocytes showed increased expression of many tumor suppressor and oncogenes, approximately at the same levels. However, in the pulse+chase experiment, the expression of oncogenes was strongly diminished in the Sirt1-Tg pneumocytes, whereas tumor suppressors suffered a significantly milder downregulation (New Figure 4F). These data indicate that the net effect of Sirt1 overexpression is anti-tumorigenic, and explains our findings in mice and human tumors. We have commented on these findings in the text.

8.- In the discussion, the authors highlight metabolic networks in the context of SIRT1. However, such discussion seems irrelevant in a manuscript where the authors did not provide any relevant data related to metabolism.
[AUTHORS] We agree with this reviewer that our data is not focused on metabolic pathways, and we have worked instead extensively in tumor pathways. We have also found several metabolic genes altered in our studies, but we have followed this reviewer's advice and removed the focus on Sirt1 metabolic functions from our Discussion section.

Minor concerns:

9.- In Figure 1B, mRNA expression of SIRT1 rebounds at day 7 in culture. This seems like an intriguing observation that worth some discussion.

[AUTHORS] We have included a remark on this point in the Discussion section: "*Interestingly, this decrease in Sirt1 protein was accompanied by a decrease in Sirt1 mRNA only in Sirt1-Tg MEFs, indicating a stronger pressure by K-Ras-activation towards Sirt1 decrease specifically on Sirt1-overexpressing MEFs. Sirt1 mRNA levels rebound slightly after 7 days in culture (Figure 1B), which could indicate a compensatory mechanism for the decreased Sirt1 protein levels and stability observed.*"

10.- In Figure 2C, some of the western blots for pERK, pAKT, and pFOXO are of poor quality. The authors should clearly show this by redoing western blots to prove if the inhibitor treatment worked in the conditions they used.

[AUTHORS] We apologize for the quality of some of our blots. We have improved their quality, as shown in new Figure S2C.

11.- In Figure 3, the authors injected tamoxifen to induce Cre recombinase activity for K-RasG12V KI. Please specify which Cre-ER system was used in this experiment. Some Cre-ER lines are known to be leaky, and therefore describing the line used (or confirming deletion only upon treatment) will be reassuring.
[AUTHORS] We have used the same Cre-ER system described by Dr. Mariano Barbacid's laboratory in the first report of their K-Ras-KI mouse model [1]: the Cre-RERT fusion protein described in [4], under the control of the locus encoding the large subunit of RNA polymerase II. These mice do not suffer any type of pathology unless treated with tamoxifen, which indicates that any possible leakiness by this system would be marginal.

12.- In Figure 4, the authors should check if most of the KFP+ cells have K-RasG12V activation at the same time. Different lox-stop-lox cassettes have different recombination efficiency in the presence of Cre recombinase. The authors cannot assume that most of the KFP+ cells indeed have activated K-Ras.
[AUTHORS] We fully agree with this reviewer on the importance of a careful selection of the reporter genes to select the cells of interest. Actually, we have recently published a paper on the differences between reporters placed in cis (as the LacZ reporter of our system) and placed in trans (as the Katushka reporter in our system) [5]. In this work, we concluded that reporters placed in cis are more reliable than those placed in trans, although both systems are informative. Following this line, we have sorted the K-Ras-KI pneumocytes using the LacZ reporter for the new pulse experiment.

Reviewer #3:

SIRT1 is a member of the Sirtuin family of NAD+-dependent deacetylases involved in stress response signaling. The role of Sirt1 in cancer is complex and seems to depend on the functional context as it has been shown upregulated or downregulated in a wide range of cancers. In the current manuscript by Herranz et al., the authors study a direct link between the SIRT1 and K-RAS in lung cancer. The authors observe a decrease in SIRT1 protein levels upon induction of K-RAS mutant G12V and link it to MAPK pathway signaling. Moreover, they demonstrate that the formation of lung adenocarcinomas in mice produced by K-RAS-G12V expression is delayed by SIRT1 increased levels (sirt1-tg mice). The authors show that higher levels of SIRT1 in these animals correlate with an increased lifespan in 24% and a lower rate of carcinomas. Interestingly, SIRT1 did not have any effect on the formation of lung adenomas, a previous step to formation of carcinomas, or in tumor growth once had appeared. Confirming their claims, the authors also analyze a set of NSCLCs human tumors and correlate SIRT1 increased levels with longer overall survival. To study the changes associated to SIRT1 expression, the authors perform RNAseq in isolated pneumocytes from K-RASG12V/SIRT1Tg animals and identify several cancer-related genes downregulated by SIRT1 induction.
Overall, this is a very interesting set of findings. Although the protective role of SIRT1 in cancer has been suggested before, the role of SIRT1 in lung carcinogenesis had not been well defined. Previous studies already described a functional interplay between SIRT1 and K-RAS signaling (Cheng et al, 2015), including K-RAS deacetylation by SIRT1, in established non-small cancer cells. However, these studies were entirely based on lung cancer cell lines. Interestingly, in this work the authors follow the in vivo development of lung tumors and observe that the protective effect of SIRT1 is relevant mainly in the first stages of tumorigenesis. This is a very relevant issue, as the vast majority of studies on the role of SIRT1 in tumorigenesis have been performed in established tumors or cell lines derived from them. This work may help reconcile the observation that SIRT1 is found both downregulated and upregulated in cancer as suggests that SIRT1 prevents tumor formation but may be required later for tumor development. However, there are several major issues that limit considerably the relevance of the work:

13) I have problems interpreting the data in Figures 1c and 2. The observed effect of K-RAS on SIRT1 levels is mild and the authors should be very careful about drawing conclusions from these experiments. In Figure 1c, the only clear conclusion is that the profile of SIRT1 levels between K-RAS WT or G12V is different at day 4. This could be relevant for the claims of the authors as it

supports a differential effect of both K-RAS forms towards SIRT1 in early stages of K-RAS induction (between 0-4 days). Unfortunately, it is based on a single point (4d) and should be developed further to make a convincing claim. One possibility would be to also show the levels of SIRT1 between day 1-4. A second possibility would be to demonstrate this effect through another approach. If the authors claim that K-RAS mutant inhibits SIRT1 more than WT at the protein level, the half life of SIRT1 protein should be different. To test that, the authors could study SIRT1 stability under these conditions around 4 days by Cycloheximide (CHX) treatments.
[AUTHORS] Following the suggestion of this reviewer, we have performed a careful study of Sirt1 protein stability using cycloheximide. These studies have indeed shown that time in culture or K-RasG12V activation reduced Sirt1 protein stability, and that treatment with MEKi reverted this effect (New Figures 1D, 2B and 2C). We consider that this suggestion has helped us to strongly reinforce our mechanism of Sirt1 regulation by K-Ras.

1) In the same line, in Figure 2 the effect of MEKi or PI3Ki treatment on SIRT1 levels is quite different between replicates. This makes difficult to draw any clear conclusion, particularly in fig 2B quantification, as the levels of SIRT1 between DMSO, MEKi or PI3Ki do not seem to be statistically significant at day 4. The authors should provide more duplicates to improve statistics. Additionally, an alternative possibility could also be to perform CHX studies with these treatments. If included, this would strengthen considerably the claims of the manuscript.
[AUTHORS] We have validated our initial observations of Sirt1 decrease in MEFs using a different model, namely, K-Ras-KI MEFs infected with Adeno-Cre. As shown in New Figure 2D, we also observed a significant decrease in Sirt1 protein levels after Adeno-Cre infection, that was reverted by a treatment with MEKi. We consider that these new experiments strongly reinforce our initial observations with time in culture. Also, following this reviewer's suggestion, we have tested Sirt1 protein stability by CHX experiments with time in culture and with K-RasG12V activation, and have observed that both stimuli reduced Sirt1 protein stability, as shown in New Figures 1D, 2B and 2C. Again, we thank this reviewer because these suggestions have greatly contributed to strengthen the mechanism of our paper.

2) In Figure 1c experiments, is there any difference in the proliferation rate of K-RAS-KI between WT and Tg SIRT1? Additionally, does either inhibition (e.g Ex-527) or downregulation of SIRT1 alter K-RAS-KI induced proliferation? Any data in this direction would strengthen the antagonism between K-RAS- G12V and SIRT1.
[AUTHORS] As reported before [1], K-RasG12V activation does not induce any proliferative arrest in MEFs, as happens with the activation of other Ras members; instead K-RasG12V activation produces a mild increase in MEF proliferation. As shown in New Figure S1D, we also observed this reported mild increase in cell proliferation after K-RasG12V activation. In addition, we also observed that Sirt1-Tg MEFs proliferated as fast as Sirt1-WT MEFs after K-RasG12V activation. Although we did not directly reduce Sirt1 levels, we found that time in culture induces a decrease in Sirt1 protein levels, as shown in Figure 1A, and this decrease in Sirt1 protein did not elicit any reduced cell proliferation in Sirt1-WT or Sirt1-Tg MEFs. We therefore consider that reduction of Sirt1 protein in MEFs does not alter cell proliferation.

3) How do the authors explain that of all 8 cell lines tested in Fig. 2c-d, only 4 show SIRT1 upregulation? In fact, only in 2 of these lines, the upregulation of SIRT1 shows a two-fold increase. These issues should be discussed if the authors claim a general effect of SIRT1 in lung cancer. Moreover, the lack of statistical analysis in Fig. 2D is also a concern.
[AUTHORS] As explained before, we present K-Ras status in all checked cell lines (Table S1). As shown, Sirt1 levels respond to MEK and/or PI3K inhibition only in lung adenocarcinoma cell lines, and not in lines from small cell carcinomas (H841) and large cell carcinoma (H661), suggesting that this response of Sirt1 is specific to adenocarcinomas. Moreover, there are two lung adenocarcinoma cell lines that do not respond to MEKi/PI3Ki: H358 and H23. Interestingly, these cell lines harbor the same K-Ras mutation: G12C, while the other responding cell lines have either WT K-Ras (Calu3) or different mutations of K-Ras (G12S for A549 and G12V for H441). This is interesting, since different mutations in K-Ras have been associated to different prognostic and molecular features. We have included a comment on this issue in the text.

4) As stated by the authors, several studies suggested that SIRT1 expression actually associates with poor prognosis (Noh et al., 2013; Grbesa et al, 2015; Lin and Peng, 2016). The authors explain the discrepancy because these studies seemed to analyze mainly squamous cell carcinomas rather than

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adenocarcinomas. However, at least another study (Li et al, Onco Targets Ther, 2015) makes that claim directly on adenocarcinomas. These studies contradict the results in figure 3F. The authors should discuss these differences.

[AUTHORS] We have included a comment on this contradiction in the text, referring to all the publications indicated by this reviewer, and acknowledging that "*This discrepancy can be explained by different patient populations, different set of driver mutations present in the tumors, or other technical differences*".

References for reviewers.

1. Guerra C, Mijimolle N, Dhawahir A, et al (2003) Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell 4:111–20.
2. Loonstra A, Vooijs M, Beverloo HB, et al (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc Natl Acad Sci U S A 98:9209–14. doi: 10.1073/pnas.161269798
3. Bowden DH (1983) Cell turnover in the lung. Am Rev Respir Dis 128:S46-8. doi: 10.1164/arrd.1983.128.2P2.S46
4. Brocard J, Warot X, Wendling O, et al (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. Proc. Natl. Acad. Sci. 94:
5. Sánchez-Luengo MÁ, Rovira M, Serrano M, et al (2017) Analysis of the advantages of cis reporters in optimized FACS-Gal. Cytom Part A 91:721–729. doi: 10.1002/cyto.a.23086

2nd Editorial Decision 23 May 2018

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 enclosed below).

As you will see, both referees support the publication of your manuscript in EMBO reports. However, both have minor further suggestions/concerns, we ask you to address in a final revised version of your manuscript.

Further, I have the following editorial requests:

Please provide the abstract written in present tense.

Please add a short running title to the title page of the manuscript (of no more than 40 characters including spaces).

We will publish your manuscript as short report. Therefore, please combine the results and discussion sections to one section termed "results and discussion".

Please upload editable TIFF or EPS-formatted single figure files in high resolution also for the four EV figures. Also upload the EV table as separate file. Please name this table "Table EV1", and update the callouts in the manuscript file. Finally, please add the legends for EV figures and table to the main manuscript file (below the legends for the main figures).

As all Western blots have been significantly cropped, would it be possible to obtain the original source data for these, 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. Please submit the source data (scans of the entire blots) shown in the main and EV figures, include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

There is a callout for Fig. 1E in the manuscript text, but there is no panel labeled 1E in Fig. 1. There is no callout for panel 2G. Please correct this.

Please add author Claudia Vales-Villamarin to the author contributions, and indicate her

contribution.

Please remove the paragraph "for more information" on the current page 31 of the manuscript.

Please provide information about the deposition of the RNA-seq data (database, accession number).

When submitting your revised manuscript, we will require:

- a letter detailing your responses to the remaining 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 if changed, and all the EV figures)

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 about 400 pixels) that can be used as part of a visual synopsis on our website.

Please note that we now mandate that all corresponding authors list an ORCID digital identifier! Please provide the ORCID of authors Serrano and Herranz, and link these to their EMBO reports profiles.

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 #2:

In this revised version of their manuscript, Fernandez-Marcos and colleagues did major efforts to address the reviewers' concerns, and the manuscript is indeed highly improved, particularly the new MEKi experiments (Fig.2) and the new RNA-seq experiments (Fig.4). I have only one comment:

- Given that, indeed, only primary MEFs exhibit the decrease Sirt1 expression, while immortalized ones do not, it is then unclear whether Ras itself decreases SIRT1, or it is actually senescence-induced stress that causes the change of Sirt1, in which case the mechanisms driving such downregulation remains unclear. Although the experiments where K-Ras is deleted provides some support that such downregulation could partially be responsible, the authors may want to change the subheading ("Sirt1 protein levels decrease in culture through a K-Ras-mediated mechanism") since it is not accurate.

Referee #3:

The new evidence included in this revised version has strengthened considerably the authors' claims and have addressed the majority of my previous concerns. The effect of K-RASG12V and MEKi on Sirt1 stability is convincing and supports the authors' model. I only have three remaining issues:

1) In Figure 2 I am not sure I understand the difference between 2E and F/G. Is this the same experiment? If this is the case, I would suggest to fuse 2E and G by representing 2E like 2G adding statistics. I would also recommend to leave Fig 2F the way it is right now, but replacing the Sirt1 WB in H441 with a higher quality image.

2) The authors observe that the effect of K-RASG12V on Sirt1 protein levels is more obvious in Sirt1-Tg MEFs than in WT MEFs, but the same happens in the case of Sirt1 mRNA. If understand it well, this suggests that a significant part of Sirt1 inhibition takes place at the level of

transcription/RNA stability. Therefore the observed Sirt1 protein levels would result from a decrease in Sirt1 stability and in transcription/mRNA levels. Although is mentioned somehow in the manuscript, I feel that this has not been really discussed despite its relevance to explain the global repressive mechanism of Sirt1 by KRAS. In the same line, a model at the end of Figure 4 would also help the authors to deliver the main message of the manuscript.

3) Page 17, line 7. "Figure 1B" is now "EV1A". This should be corrected.

2nd Revision - authors' response 7 June 2018

EMBOR-2016-43879V2
Response to Reviewer's comments.
[AUTHORS] We thank the reviewers for their very positive comments: that we "*did major efforts to address the reviewers' concerns*", that "*the manuscript is indeed highly improved*", and that "*the new evidence included in this revised version has strengthened considerably the authors' claims*".
We proceed to answer the reviewers' remaining issues:

Referee #2:

In this revised version of their manuscript, Fernandez-Marcos and colleagues did major efforts to address the reviewers' concerns, and the manuscript is indeed highly improved, particularly the new MEKi experiments (Fig.2) and the new RNA-seq experiments (Fig.4). I have only one comment:

- Given that, indeed, only primary MEFs exhibit the decrease Sirt1 expression, while immortalized ones do not, it is then unclear whether Ras itself decreases SIRT1, or it is actually senescence-induced stress that causes the change of Sirt1, in which case the mechanisms driving such downregulation remains unclear. Although the experiments where K-Ras is deleted provides some support that such downregulation could partially be responsible, the authors may want to change the subheading ("Sirt1 protein levels decrease in culture through a K-Ras-mediated mechanism") since it is not accurate.
[AUTHORS] We fully agree with this reviewer's comment: it is possible that culture-driven senescence is one of the mechanisms inducing decreased Sirt1 protein levels in cultured MEFs. Senescence is a stress response involving many signaling pathways, including K-Ras. There might be other pathways involved in senescence other than K-Ras that similarly decrease Sirt1 protein levels, which we have not studied in our work. Instead, we focused on K-Ras, and we have proved that oncogenic K-Ras activation partly recapitulates the culture-driven Sirt1 decrease, thus linking K-Ras-culture-driven senescence and Sirt1 protein decrease.

Since we fully agree that K-Ras might not be the only culture-driven stress decreasing Sirt1 protein levels, we have followed this reviewer's advice and reworded the subheading, that now reads "*K-Ras participates in the decrease of Sirt1 protein levels in culture*".

Referee #3:

The new evidence included in this revised version has strengthened considerably the authors' claims and have addressed the majority of my previous concerns. The effect of K-RASG12V and MEKi on Sirt1 stability is convincing and supports the authors' model. I only have three remaining issues:

1) In Figure 2 I am not sure I understand the difference between 2E and F/G. Is this the same experiment? If this is the case, I would suggest to fuse 2E and G by representing 2E like 2G adding statistics.
[AUTHORS] Figure 2E is the quantification of the single-lane Western blots presented in Figure EV2C. Based on this Figure, we focused our attention on the cell lines that increased their Sirt1 protein levels upon MEK or PI3K inhibition (H441, A549 and Calu3), and then performed the same treatments in triplicate. This more complete experiment is presented in Figure 2F, and quantified in Figure 2G. To make this point clearer in the text, we have explained it more carefully: *"Interestingly, inhibition of MEK or PI3K resulted in the upregulation of SIRT1 protein levels in 3*

out of 7 lung tumor cell lines (Calu3, A549 and H441, Figure 2E and EV2C), and we validated this decrease in the positive cell lines (Figure 2F-G and EV2D)".

I would also recommend to leave Fig 2F the way it is right now, but replacing the Sirt1 WB in H441 with a higher quality image.
[AUTHORS] We have inserted a higher quality image for the H441 Sirt1 Western blot. We hope this new image is clear enough.

2) The authors observe that the effect of K-RASG12V on Sirt1 protein levels is more obvious in Sirt1-Tg MEFs than in WT MEFs, but the same happens in the case of Sirt1 mRNA. If understand it well, this suggests that a significant part of Sirt1 inhibition takes place at the level of transcription/RNA stability. Therefore the observed Sirt1 protein levels would result from a decrease in Sirt1 stability and in transcription/mRNA levels. Although is mentioned somehow in the manuscript, I feel that this has not been really discussed despite its relevance to explain the global repressive mechanism of Sirt1 by KRAS.
[AUTHORS] We agree with this reviewer that K-Ras activation decreased *Sirt1* mRNA levels, but we only find this in Sirt1-Tg MEFs, and not in Sirt1-WT MEFs (see Figure EV1A and EV1E). Following this reviewer's comments, we have included an explanation of these findings: "*These findings suggest that K-Ras overcomes the described Sirt1-induced proliferative arrest by decreasing Sirt1 expression through two mechanisms: first, K-Ras induces a decreased Sirt1 transcription in the cells with high expression of Sirt1 (Figure EV1A and EV1E). Second, irrespective of Sirt1 protein levels, K-Ras induces a decrease in Sirt1 protein stability via MAPKs in MEFs, or via both MAPKs and PI3K in different human lung cancer cell lines (Figure 2)*"

In the same line, a model at the end of Figure 4 would also help the authors to deliver the main message of the manuscript.
[AUTHORS] Following the suggestion of this reviewer, we have inserted a new panel in Figure 4 (Figure 4G) describing our findings and the mechanistic model that we propose based on them.

3) Page 17, line 7. "Figure 1B" is now "EV1A". This should be corrected.
[AUTHORS] We have corrected this mistake in the text.

3rd Editorial Decision 12 June 2018

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

However, I have these further editorial requests that need to be addressed:

- Please format the references according to our journal style. If there are more than 10 authors, 'et al' should be used, but keeping the first 10 authors. See: http://embor.embopress.org/authorguide#referencesformat

- Please upload Table EV1 as a pdf with the legend on the same page. Then remove the legend for this table from the manuscript text.

- For the synopsis figure, we would require a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that depicts the major findings of your work (not just a data figure). Maybe, this can be based on Fig. 4G? It would also be fine to provide a version of Figure 4G in the dimensions mentioned above.

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 (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

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.

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3rd Revision - authors' response 18 June 2018

The authors made the requested revisions.

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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: Pablo J. Fernandez-Marcos
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43879-T

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.

USEFUL LINKS FOR COMPLETING THIS FORM

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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?	P29: For animal experiments, sample size was chosen by introducing statistical parameters from previous, similar experiments (Joshi et al. 2008), estimating a hazard ratio of 2.5 and the power of the statistical test set at 0.8. This analysis resulted in an estimation of sample size of 15-20 animals per group. For in vitro experiments, we used a minimum of 3 independente replicates, estimating that this sample size is adequate to detect robust changes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	P29: For animal experiments, sample size was chosen by introducing statistical parameters from previous, similar experiments (Joshi et al. 2008), estimating a hazard ratio of 2.5 and the power of the statistical test set at 0.8. This analysis resulted in an estimation of sample size of 15-20 animals per group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	P20: For the survival curves, mice that were sacrificed due to verified non-tumor causes (dermatitis, fighting wounds, eye soreness) were censored our from the curves. For the rest of experiments with mice, we only analyzed those animals that did not die in cage, but were sacrificed following humane end-point criteria.
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.	P29:Mice were not treated, but instead they were alocated to different groups by their genotype (Sirt1-WT or Sirt1-Tg).
For animal studies, include a statement about randomization even if no randomization was used.	P29: No randomization was used.
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.	P22 and P29: Except for the pathological analyses, investigators were not blinded to the experimental groups (cell types or mouse genotypes)
4.b. For animal studies, include a statement about blinding even if no blinding was done	P22 and P29: Except for the pathological analyses, investigators were not blinded to the experimental groups (cell types or mouse genotypes)
5. For every figure, are statistical tests justified as appropriate?	All statistical tests are justified for each figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	P29: Where possible, normal distribution of data was checked by the Shapiro-Wilk test, and equal variances were checked by the F test.

Is there an estimate of variation within each group of data?	P29: Where possible, normal distribution of data was checked by the Shapiro-Wilk test, and equal variances were checked by the F test.
Is the variance similar between the groups that are being statistically compared?	P29: Yes

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).	P20 and P23: All references and clone IDs are included in the manuscript, and all of them are validated by their respective commercial companies. Where available, we have checked in the indicated databases and confirmed their validation for the system under study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	P19: Human lung tumor cell lines were purchased from the ATCC and periodically tested for mycoplasma.

* 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.	P20: Animal experimentation at the CNIO, Madrid, was performed according to protocols approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CEIyBA). Mice (Mus musculus) of both sexes and mixed background were generated by crossing K-Ras+/LSLG12Vgeo mice (Guerra et al, 2003) with Sirt1Tg mice (Pfluger et al, 2008) and Katushka-KI mice (Diéguez-Hurtado et al, 2011).
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.	Compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	P22: Primary lung tumors were collected and handled anonymously at collaborating institutions (Instituto Angel H. Roffo and Hospital Britanico) after approval by their Institutional Review Boards (IRB)
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.	P22: Human samples were collected following standard ethical and legal protection guidelines of human subjects, including informed consent. Experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
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.	P23:Compliance confirmed

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	Data from RNAseq will be deposited in public databases.
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).	OK
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