EMBO Molecular Medicine

NRAS destines tumor cells to the lungs

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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.)

Editor: Céline Carret

1st Editorial Decision 23 September 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript. Although the referees find the study to be of potential interest, they also raise a number of concerns that need to be addressed in the next final version of your article.

As you will see from the comments pasted below, both referees are supportive of publication. While referee 2 has minor comments and suggests further clarifications here and there, referee 1 would like to see a better metastasis model *in vivo*, and additional experiments using shRNAs to provide controls and mechanism.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please read below for important editorial formatting for submission of your revised article.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks):

Recent data point out that specific mutations in primary tumors could determine the site of metastatic invasion. This is new and very interesting concept in the metastasis biology that should be further explored. In this context, the results presented in this manuscript can help us better understand the possible association of N-Ras mutations in primary tumor with lung metastasis development. The data provided by the Authors is interesting and suggests that N-Ras mutants facilitate lung colonization by controlling the expression of IL-8 family chemokines, which impinge on the niche by modifying the lung endothelium and myeloid cell recruitment. The manuscript is concise, well written with solid and well presented data. However, there are some concerns that should be addressed.

Major points
1. My main concern is the use of lung tumor cell lines implanted subcutaneously to address the lung metastasis. This is an unlikely setting since contralateral lung mets are more typical. In addition, the prevalence of NRAS mutations in lung cancers is far less frequent than KRAS mutations and the lung cancer cell lines chosen carry both of these mutations. Thus, the central question is to what extent the effect detected is NRAS and not KRAS driven? In line with that, the authors only use one short hairpin RNA, and off target effects are a risk. Given that Ras genes present a significant sequence homology data regarding short-hairpin specificity is a must. Is expression of H-Ras or K-Ras affected by this short hairpin RNA? Additionally, a second independent shorthairpin against NRAS and not KRAS is needed at least for key experiments.

2. Based on the data from WBs presented in this manuscript the levels of NRas are significantly higher in AE17 and LLC cell lines comparing to MC38 cell line. Could it be that simply N-Ras overexpression and not N-Ras mutation is what actually facilitates lung colonization? Or a synergism between both effects drives the effect? The authors could overexpressing N-Ras wild type/mutant isoform in MC38 cell line and test which of these two conditions contributes to lung metastasis formation. What are the N-Ras levels in all cell lines presented in Figure 1A? Is there any difference in Ras downstream pathway activation between these cell lines?

Minor comments:
1. The sentence in abstract "In conclusion, mutant NRAS promotes the colonization of the lungs by various tumor types" seems an unnecessary overstatement as the mechanistic studies presented were obtained with mouse lung cancer cell lines. Similarly, in the discussion section there are unnecessary overstatements.

2. Previous evidence that the mutational status may influence the site of metastasis is shown in the literature, particularly in KRAS (i.e. Urosevic et al NCB 2014 and others). Please cite or modify the introduction accordingly (line 7 and 8 of 2 paragraph, page 3)

3. Please provide the data on primary tumor size for Figures 2A,B, C, D and 3F. Please explain how in 4 weeks metastatic bioluminescent signal of lungs could be stronger than that detected in primary tumor as shown in Figure 2A?

Referee #2 (Comments on Novelty/Model System):

The authors have used a variety of different human and mouse cells lines as well as several engineered animals models to validate their findings.

These findings have the potential to be further tested in clinical trials to reduce metastases in patients with Nras mutant tumors.

Referee #2 (Remarks):

In this study, Giannou and colleagues elegantly show that a NRASQ61K mutation promote lung metastasis by facilitating the extravasation of circulating tumor cells into the lung parenchyma. They go on and show that interleukin 8 related chemokine expression is responsible to initiate a communication between tumor cells, the pulmonary vasculature and myeloid cells. By suppressing chemokine signaling with Navarixin (a CXCR1/2 inhibitor) they were able to reduce lung colonies, suggesting that this therapy might prevent lung metastasis in patients with NRAS mutant primary tumors.

The authors provide a set of experiments that make an important contribution to the field of pulmonary metastasis and will be of broad interest. The experiments are very well designed and executed and the number of mice and the different strains used are impressive, significantly strengthening the conclusions of the manuscript.

Although no major revisions are needed, addressing the following comments and a more comprehensive presentation of the data (several times information within the text is missing) will strengthen the manuscript.

Minor comments:
1. Figure 1 misses the appropriate reference in the text when refereeing to the different panels. It will help the reader to follow each individual panel with a text reference. Some information could be moved to supplementary figures, ie: panel B is not needed in the main figure.

I am somehow puzzled that tumors were grown to almost 5cm3 when current legislation in Europe does not allow volume larger than 1.5 cm3. Was this needed to obtain metastasis?

2. Figure 2C. It is very difficult to see the lung figure on its current form. Are these confocal images? A higher magnification is suggested to better appreciate the stainings. At what time point were the pictures taken? I fail to see metastasis in these lungs.

It is not clear why MC38 cell line was chosen for the experiments in figure 2. Why not choosing a cell line that develops metastasis although at a lower rate without the Nras mutation? Like A549 or B16F10?

3. Figure 3A, B and C panels are confusing. What does it mean X MC38 on the Y axis on panel A? and % shC in panel B? A clearer way to represent the data of the QPCR would help the reader. The same will apply to figure 4. Further description on the quantification in the supplemental material should be provided.

Also not clear is panel C. The knock down in AE17 seems much better than in LLC, however the RNA data shows only 50% reduction in AE17 compared to 80% reduction in LLC.
Figure 3E shows tumor growth s.c of about 1.5cm3 however figure 1C shows an average tumor volume of 4cm3 after 30 days for the same cell line. Is the discrepancy in the size responsible for the lower number of metastasis?

Figure 3G, MC38 was able to form forced metastasis when injected I.V. although with less efficiency than LLC and AE17. It is not clear why these numbers as well as HE pictures of the lungs were not included in figure 3G.

Figure 3H, could the authors comment why they choose to transfect HEK293T cells with a plasmid encoding NRASG61K and not any other of the cells lines presented in figure 1A, for which the mutational analysis was performed? In fact, MC38 was engineered to express pANRAS (see figure 4C) however the number and pictures of the lungs/metastasis was not provided.

4. They authors nicely show that NRAS mutation leads to the up-regulation of Cxcl5 (in mouse cell lines) or Cxcl6 (in human). I was wondering if the chemokine induction is the cause of metastatic colonization or the NRAS mutation per se. Could the authors show if overexpression of Cxcl5 or 6 in the absence of NRAS mutations leads to increase metastasis?

RESPONSE TO REVIEWERS' COMMENTS

Referee #1

"Recent data point out that specific mutations in primary tumors could determine the site of metastatic invasion. This is new and very interesting concept in the metastasis biology that should be further explored. In this context, the results presented in this manuscript can help us better understand the possible association of N-Ras mutations in primary tumor with lung metastasis development. The data provided by the Authors is interesting and suggests that N-Ras mutants facilitate lung colonization by controlling the expression of IL-8 family chemokines, which impinge on the niche by modifying the lung endothelium and myeloid cell recruitment. The manuscript is concise, well written with solid and well presented data. However, there are some concerns that should be addressed."

We sincerely thank this Reviewer for her/his positive overall appraisal of our work. We have undertaken every effort feasible to address her/his critiques.

"1. My main concern is the use of lung tumor cell lines implanted subcutaneously to address the lung metastasis. This is an unlikely setting since contralateral lung mets are more typical."

We thank the Reviewer for the pertinent comment. We did not examine lung cancer cell lines only, but a variety of murine and human cell lines of pulmonary, pleural, skin, enteric, mammary, and other origins. This is why we chose the skin heterotopic model, which features automatic, spontaneous, physiologically relevant metastasis. This model is also used by leading authorities in the field (Acharyya et al., 2012; Chen et al., 2011; Lyden et al., 2001; Qian et al., 2011). We could not have used any orthotopic model to compare side-by-side the metastatic propensities of these cells, because this would bias the results, since the local microenvironment would be syngeneic to some tumor cell lines only and alien to others. This issue is now clarified in the revised introduction and results sections.

"In addition, the prevalence of NRAS mutations in lung cancers is far less frequent than KRAS mutations and the lung cancer cell lines chosen carry both of these mutations."
Again we would like to stress that this study was not about lung cancer metastasis only, but was designed to mainly address lung-directed metastasis of cancer cells of any origin. Although *NRAS* mutations in lung cancer are infrequent, they do exist. Maybe it is these lung cancer that produce satellite nodules in the lung themselves? This will hopefully addressed by future clinical studies that are designed appropriately to tackle this question.

"Thus, the central question is to what extent the effect detected is NRAS and not KRAS driven? In line with that, the authors only use one short hairpin RNA, and off target effects are a risk. Given that Ras genes present a significant sequence homology data regarding short-hairpin specificity is a must. Is expression of H-Ras or K-Ras affected by this short hairpin RNA? Additionally, a second independent shorthairpin against NRAS and not KRAS is needed at least for key experiments."

This is a key concern and we thank the Reviewer for pointing this out. We are sorry these issues were not clear enough in the initial submission. We used pools of three different shRNAs against NRAS, and not a single shRNA. These were provided as lentiviral pools by the vendor, so we could not use them separately. This would also increase the number of mice used for these studies, which is already very high (n = 611). To alleviate the Reviewer's valid concern, we now undertook new experiments using shRNA pools against *Kras* obtained from the same vendor and compared *Kras* and *Nras* silencing effects on primary tumor growth, pulmonary metastasis, and on NRAS and KRAS expression at the RNA levels in new Figure 4. In addition, we graphically provide the shRNA target sequences of the two different shRNA pools on the *Nras* and *Kras* transcripts in new Figure EV3. Our new results show that the shRNA pools used are specific and function to specifically silence the respective target transcripts without significant off-target effects on the undesired target transcript. In detail, *Nras* silencing decreased *Nras* but not *Kras* RNA levels in LLC and AE17 cells, while *Kras* silencing decreased *Kras* but not *Nras* RNA levels in LLC and AE17 cells (new Figure 4A). Moreover, that *Kras* signaling functions to promote both tumor growth and pulmonary metastasis, while *Nras* seems to more specifically promote lung metastasis, since it does not profoundly impact tumor growth, but is pivotal for pulmonary metastasis (new Figures 4C,

EV2H and EV2I). The Reviewer's concern, the relevant new experiments, and the revised interpretation of the data taking KRAS into account are now included in the revised manuscript.

"2. Based on the data from WBs presented in this manuscript the levels of NRas are significantly higher in AE17 and LLC cell lines comparing to MC38 cell line. Could it be that simply N-Ras overexpression and not N-Ras mutation is what actually facilitates lung colonization? Or a synergism between both effects drives the effect? The authors could overexpressing N-Ras wild type/mutant isoform in MC38 cell line and test which of these two conditions contributes to lung metastasis formation."
The Reviewer is again correct, since RAS protein expression levels are of pivotal importance in addition to mutations (Stephen et al, 2014). To address this, we obtained a wild-type-NRAS encoding NRAS plasmid and overexpressed wild-type as well as Q61K-mutant NRAS in MC38 cells and injected stable clones s.c. The results, shown in new Figures 3B-3D and EV2F, indicate that either a mutation or elevated levels of wild-type NRAS are required for the observed phenotype of NRAS-mutant cells. The pertinent point of this Reviewer is also discussed in the revised Discussion section and changes were made throughout the manuscript, including title and abstract, to account for this point.

"What are the N-Ras levels in all cell lines presented in Figure 1A? Is there any difference in Ras downstream pathway activation between these cell lines?"

qPCR and immunoblot data of NRAS expression and of activation of RAS-downstream signaling pathways ERK, MAPK, NF-κB, CREB, STAT, and NOTCH are now shown in new Figure EV1. The results indicate that, as this Reviewer and other authorities suspected, mutation is intrinsically associated with overexpression and overactivation of downstream signaling pathways. The new results are now discussed in the revised results and discussion sections.

"1. The sentence in abstract "In conclusion, mutant NRAS promotes the colonization of the lungs by various tumor types" seems an unnecessary overstatement as the mechanistic studies presented were obtained with mouse lung cancer cell lines. Similarly, in the discussion section there are unnecessary overstatements."
The sentence in question was rephrased to "In conclusion, *NRAS* promotes the colonization of the lungs by various tumor types in mouse models". Similar unnecessary overstatements in the discussion section were also amended or rephrased.

"2. Previous evidence that the mutational status may influence the site of metastasis is shown in the literature, particularly in KRAS (i.e. Urosevic et al NCB 2014 and others). Please cite or modify the introduction accordingly (line 7 and 8 of 2 paragraph, page 3)"

The relevant reference was included and the corresponding phrase was rephrased to "However, these signatures are hard to target, and direct links between a single cancer mutation and metastatic tropism to a given organ, such as those suggested by observational studies of *NRAS* and *KRAS* mutations in pulmonary and hepatic metastasis of colon cancer and melanoma (Lan et al, 2015; Urosevic et al, 2014; Tie et al, 2011; Ulivieri ey al, 2015; Pereira et al, 2015), are intriguing and of potential clinical value".

"3. Please provide the data on primary tumor size for Figures 2 A, B, C, D and 3F. Please explain how in 4 weeks metastatic bioluminescent signal of lungs could be stronger than that detected in primary tumor as shown in Figure 2A?"

We are sorry to have mistakenly failed to identify in the legend and relevant text to Figures 2A and 2C that, for the purposes of these experiments, the primary tumors were either removed after two weeks and mice were allowed to survive (2A), as the primary signal largely overshadowed the metastatic signal, or were prematurely killed (2C). In the images shown in Figure 2A, the primary tumor signal stems from residual tumor tissue. This was corrected in the revised version of the manuscript. We are truly sorry for this mistake. Primary tumor size data for all main Figures are now included in new Figure EV2. However, we stress that, in all experiments except the ones shown in Figures 2A and 2C, mice were observed till moribund without intervention, in order to observe spontaneous, physiologically relevant metastasis.

Referee #2

"The authors have used a variety of different human and mouse cells lines as well as several engineered animals models to validate their findings. These findings have the potential to be further tested in clinical trials to reduce metastases in patients with Nras mutant tumors. In this study, Giannou and colleagues elegantly show that a NRASQ61K mutation promote lung metastasis by facilitating the extravasation of circulating tumor cells into the lung parenchyma. They go on and show that interleukin 8 related chemokine expression is responsible to initiate a communication between tumor cells, the pulmonary vasculature and myeloid cells. By suppressing chemokine signaling with Navarixin (a CXCR1/2 inhibitor) they were able to reduce lung colonies, suggesting that this therapy might prevent lung metastasis in patients with NRAS mutant primary tumors. The authors provide a set of experiments that make an important contribution to the field of pulmonary metastasis and will be of broad interest. The experiments are very well designed and executed and the number of mice and the different strains used are impressive, significantly strengthening the conclusions of the manuscript. Although no major revisions are needed, addressing the following comments and a more comprehensive presentation of the data (several times information within the text is missing) will strengthen the manuscript. "

We sincerely thank this Reviewer for her/his positive appraisal of our work. We really appreciate her/his constructive criticism and have undertaken every effort feasible to address her/his critiques.

"1. Figure 1 misses the appropriate reference in the text when refereeing to the different panels. It will help the reader to follow each individual panel with a text reference. Some information could be moved to supplementary figures, ie: panel B is not needed in the main figure."

We sincerely apologize and corrected this reviewer's pertinent comment. The description of Figure 1 in the results section was improved with appropriate callouts for each panel. Panel B and primary tumor growth data were moved to new Figures EV1A and EV1B to improve the legibility of Figure 1.

"I am somehow puzzled that tumors were grown to almost 5 cm^3 when current legislation in Europe does not allow volume larger than 1.5 cm^3. Was this needed to obtain metastasis?"

The Reviewer is again absolutely right. We obtained specific permits to let tumors grow out to 4-5 cm^3 and to sacrifice animals only when moribund, because the phenomenon of spontaneous metastasis occurred after tumors grew to the EU limit of 1.5 cm^3. Although waiting till these extreme tumor volumes were obtained was rarely necessary (we observed such tumor volumes only in 10 out of 611 mice used in these studies), this freedom to operate was necessary to conduct these studies since lung metastasis is a late manifestation of cancer. This is clarified in the revised Methods section and was properly addressed and approved in the animal care approvals obtained for these studies.

"2. Figure 2C. It is very difficult to see the lung figure on its current form. Are these confocal images? A higher magnification is suggested to better appreciate the stainings. At what time point were the pictures taken? I fail to see metastasis in these lungs."

This reviewer is absolutely right. We sacrificed these animals prematurely (at two weeks post-tumor cell injections) and failed to state so in the text and the Figure legend of the initial submission. We are extremely sorry for this. The aim was to identify whether tumor and myeloid cells co-segregate in space in the pre-metastatic niche. Again we apologize and have corrected these aspects. These images were taken on a regular inverted microscope (Zeiss Axio Observed D1) not equipped with confocal capabilities. Confocal images of metastasis-seeded lungs with infiltrating myeloid cells expressing CXCR2 are shown in Figure 6D. The lung structure is not easily comprehended on such images as opposed to light microscopic images, even at higher magnification. We tried to improve the reader's orientation by labeling some alveoli and bronchi in these images. We apologize to the Reviewer for the poor quality of these images, but ask for understanding, because these samples are two years old and have lost their intrinsic fluorescence, hence these images could not be replaced at this time.

"It is not clear why MC38 cell line was chosen for the experiments in figure 2. Why not choosing a cell line that develops metastasis although at a lower rate without the Nras mutation? Like A549 or B16F10?"

We thank the Reviewer for her/his sharp comment. The MC38 cell line was chosen because it is an adenocarcinoma that is syngeneic to the *C57BL/6* mouse like LLC and AE17 cells and has a *Kras* mutation, alike the LLC and AE17 cells that feature both *Nras* and *Kras* mutations. A549 cells could not be used because they are human and need immunocompromized mice. B16F10 cells seed all organs with micrometastases (bullet-like pattern) without predilection but mice die from the primary tumor before the metastatic tumor cells can grow out (enhanced primary tumor growth rate compared to all other cell lines). In addition B16F10 cells do not have any of the mutations we looked for, including the *Kras* mutation, so they would be an incomplete control. In addition, we believe that MC38 cells are very hard controls for these studies, because they display aggressive tumor growth in the flank that is comparable to the two lung-metastatic lines as shown in new Figure EV2, and because they are *Kras*-mutant as the two lung-metastatic lines, so using them we controlled for *Kras* status.

"3. Figure 3A, B and C panels are confusing. What does it mean X MC38 on the Y axis on panel A? and % shC in panel B? A clearer way to represent the data of the QPCR would help the reader. The same will apply to figure 4. Further description on the quantification in the supplemental material should be provided."

These issues, correctly pointed out by this Reviewer, were corrected throughout. x MC38 means times MC38 (was corrected and explained in the legend) and % shC means percent of corresponding control shRNA (was explained in the legend). qPCR quantification method details are now given in the Methods.

"Also not clear is panel C. The knock down in AE17 seems much better than in LLC, however the RNA data shows only 50% reduction in AE17 compared to 80% reduction in LLC."

We cannot explain this discrepancy, but we are confident of the results shown, since experiments were redone three independent times. RNA and protein level silencing efficiencies were not consistent. However, we show beyond doubt in new Figure 4A and 4B that shRNA silenced NRAS and not KRAS at both the RNA and the protein level. One explanation for the divergent RNA and protein data would be different degrees of codon bias (J Clin Invest 2015;125:222–233, Current Biol 2013;23:70–75) in LLC and AE17 cells. For example, a greater scarcity of rare codons (which exist in NRAS) in AE17 cells would explain greater protein knock-down, even with less shRNA efficiency in terms of RNA silencing. We had no space to touch upon these issues in the revised manuscript, but would be happy to do so if the Reviewer wishes.

"Figure 3E shows tumor growth s.c of about 1.5cm3 however figure 1C shows an average tumor volume of 4 cm3 after 30 days for the same cell line. Is the discrepancy in the size responsible for the lower number of metastasis?"

Yes, exactly. We believe that this reduced tumor growth is due to the shRNA transfections and the *in vitro* puromycin treatment of the shRNA stable clones. Even immediately before injections *in vivo*, shRNA expressing stable clones necessitated low concentrations of puromycin in order for selective pressure to be maintained (1 μg/mL). In addition, flank tumor growth was not always the same for a given cell line and we observed significant variation over time, even taking the outmost care in order to exactly recapitulate experiments. This Reviewer is again right: the lower metastasis number is associated with a reduced primary tumor growth rate in this case. To give a comparative overview of all flank experiments, they were now grouped together in new Figure EV2.

"Figure 3G, MC38 was able to form forced metastasis when injected I.V. although with less efficiency than LLC and AE17. It is not clear why these numbers as well as HE pictures of the lungs were not included in figure 3G."

These numbers and images were now included in the revised version (new Figure EV4D). MC38 can form forced metastases but to a lesser extent than *NRAS* mutant cells in our hands.

"Figure 3H, could the authors comment why they choose to transfect HEK293T cells with a plasmid encoding NRASG61K and not any other of the cells lines presented in figure 1A, for which the mutational analysis was performed? In fact, MC38 was engineered to express pANRAS (see figure 4C) however the number and pictures of the lungs/metastasis was not provided."
We now show the data from mutant (and wild-type) *NRAS*-expressing MC38 cells in new Figure 3D. We also included wild-type *NRAS* plasmid-expressing MC38 cells, to see whether overexpression *per se* or mutation is necessary for metastasis, according to the criticism of another Reviewer. The results indicate that a mutation is required for the observed phenotype of *NRAS*-mutant cells, and not just elevated levels of wild-type *NRAS*. The HEK293T cells were chosen because they are not malignant and tumorigenic, but merely transformed. So we garnered that, if mutant *NRAS* can render even these cells spontaneously metastatic, this would be the hardest evidence ever that *NRAS* is a pro-metastasis gene. Indeed, as shown in new Figures 3E and EV2G, mutant NRAS-expressing HEK293T cells not only form late primary tumors (as do by the way control-transfected cells), but metastasize spontaneously to the lungs.

"4. The authors nicely show that NRAS mutation leads to the up-regulation of Cxcl5 (in mouse cell lines) or Cxcl6 (in human). I was wondering if the chemokine induction is the cause of metastatic colonization or the NRAS mutation per se. Could the authors show if overexpression of Cxcl5 or 6 in the absence of NRAS mutations leads to increase metastasis?"

We thank the Reviewer for this critical comment. To test this, we undertook additional *in vivo* experiments that unfortunately failed technically. Since we could not unequivocally show that CXCR1-cognate chemokines (in this case *Cxcl5*) promote pulmonary metastasis and are the NRAS-driven culprits of lung colonization, we address this in the revised discussion section.

2nd Editorial Decision 22 February 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending final editorial amendments.

Please submit your revised manuscript within two weeks.

I look forward to reading a new revised version of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):
The authors have addressed my main technical concern. Although indirectly, the evidence seem to confirm the suitability of their model.

Referee #1 (Remarks):
The authors have provided reasonable answers to most of the points raised. In particular and central to the previous limitations are the experiments focusing on the contribution of NRAS versus NRAS mutated. In other words, they clarify that it is the amount of NRAS and not just the mutation what drives the lung colonization phenotype. In addition, although no direct data is provided the experiments where NRAS is depleted while KRAS mutation is present indirectly clarify the contribution of each of the RAS isoforms. Overall, the manuscript has significantly improved through the revision process. Although, some remaining questions are still open, the clarifications to the most important points seem satisfactory and the work relevant for the community.

Referee #2 (Comments on Novelty/Model System):
The authors have included new experiments in the revised version to address the concerns raised by this reviewer. I am satisfied with the revised version.

Referee #2 (Remarks):

This reviewer is pleased to see the new data added by the authors, which substantially improved the overall message of the manuscript and is fully satisfied with the revised version submitted.

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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: Georgios T. Stathopoulos
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2016-06978-V3

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	An a priori target sample size of n = 10/group was calculated using G*power (http://www.gpower.hhu.de/) assuming α-error = 0.05, β-error = 0.95, and Cohen's d = 1.5 resulting from 30% differences between groups featuring SD spanning 20-30% of the mean. PAGE MS-26
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	An a priori target sample size of n = 10/group was calculated using G*power (http://www.gpower.hhu.de/) assuming α-error = 0.05, β-error = 0.95, and Cohen's d = 1.5 resulting from 30% differences between groups featuring SD spanning 20-30% of the mean. PAGE MS-26
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded. PAGE MS-26
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.	Since in our animal study the sample size is relatively low as compared with clinical studies, randomization has limited applicability. Instead, we used simple alternation of littermates to treatments or interventions. After alternation and before experiments were initiated, baseline characteristics of the experimental groups (i.e., sex, body mass) were analyzed to ensure there is no statistically significant difference between the experimental groups at baseline. PAGE MS-26
For animal studies, include a statement about randomization even if no randomization was used.	Since in our animal study the sample size is relatively low as compared with clinical studies, randomization has limited applicability. Instead, we used simple alternation of littermates to treatments or interventions. After alternation and before experiments were initiated, baseline characteristics of the experimental groups (i.e., sex, body mass) were analyzed to ensure there is no statistically significant difference between the experimental groups at baseline. PAGE MS-26
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.	Data acquisition was blinded on samples previously coded by a non-blinded investigator. PAGE MS-26
4.b. For animal studies, include a statement about blinding even if no blinding was done	Data acquisition was blinded on samples previously coded by a non-blinded investigator. PAGE MS-26
5. For every figure, are statistical tests justified as appropriate?	Yes. PAGES MS-42-52.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All data were examined for normality by Kolmogorov-Smirnof test and were normally distributed. PAGE MS-26
Is there an estimate of variation within each group of data?	Values are given as mean ± SEM. PAGE MS-26
Is the variance similar between the groups that are being statistically compared?	Yes. PAGE MS-26.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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).	Yes, we provide catalogue numbers, providers, and dilutions. APPENDIX SUPPLEMENTARY TABLE S6, PAGE S8.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used are identified by source and/or citation. PAGE MS-17. Cell lines were tested annually for identity (by the short tandem repeat method) and for Mycoplasma Spp. (by PCR). METHODS PAGE 17-18.

* 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.	C57BL/6 (#000664), BALB/c (#001026), NOD/SCID (#001303), CAG.Luc.eGFP (#008450), mT/mG (#007676), Vav.Cre (#008610), Cxcr1-/- (#005820) and Cxcr2+/- (#006848) mice were from Jackson Laboratories (Bar Harbor, MN) and were bred at the Center for Animal Models of Disease of the University of Patras. PAGE MS-20.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experiments were carefully designed and approved a priori by the Veterinary Administration of the Prefecture of Western Greece (approval protocol numbers 3741/16.11.2010, 60291/3035/19.03.2012, and 118018/578/ 30.04.2014), and were conducted according to Directive 2010/63/EU (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF). Experimental mice were sex-, weight (20-25 g)-, and age (6-12 week)-matched; both male and female mice were used. PAGES MS-20.
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.	Yes

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 accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Microarray data were deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/; Accession ID: GSE74309) and pCAG.Luc plasmid with Addgene (#74409). PAGES MS-20, 21, 28.
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).	Microarray data were deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/; Accession ID: GSE74309) and pCAG.Luc plasmid with Addgene (#74409). PAGES MS-20, 21, 28.
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Yes. Primary metastasis data of human cancers were from: Disibio G & French SW (2008) Metastatic patterns of cancers: results from a large autopsy study. Arch Pathol Lab Med 132: 931-939 NRAS gain-of-function frequency data in the various human cancer types were retrieved from the Catalogue of Somatic Mutations in Cancer (http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=NRAS#dist; last accessed on 09/10/2016): Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U, Campbell PJ (2015) COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res 43: D805-811. PAGE MS-21
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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