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AMPK promotes survival of c-Myc positive melanoma cells by suppressing oxidative stress

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 July 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the reports on your work are rather mixed, with referee #1 not supporting further consideration here, while referee #2 and #3 find the provided link to AMPK-dependent survival potentially interesting. However, this link is underdeveloped as noted by all three referees, and a significant amount of new experimental data would need to be added to substantiate it. We'd normally judge the further insight requested by the referees beyond the scope of a revision of 3-5 months, but if it is feasible for you to add all requested controls and to add the requested further insight in a comprehensive manner to better support the link to AMPK dependent survival mechanistically, we are open to consider a revised version. Note that the clinical relevance of the findings for better treatment options does not need to be established (referee #3, point 1).

Should you be able to address the criticisms, I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.embo.org/embo-press

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Kfoury et al. reports a role for MYC in NRAS-driven Ink4a null melanoma, particularly regarding aggressive and metastatic disease that is dependent on AMPK activity. The authors used genetically engineered mouse models and cells to glean the role of MYC, which is known to play a critical role in cellular growth metabolism and also previously shown to underpin the tumorigenic potential of KRAS (hence RAS/RAF/MEK pathways) in a transgenic lung cancer model. As such, this specific role of MYC downstream of RAS is not a new concept. However, the authors did probe the role of AMPK and found that high MYC-mediated melanomas are dependent on AMPK activity such that loss of function of AMPK resulted in cell death, presumably due to excessive redox stress. The source of redox stress, however, is undefined. Here, it should be noted that AMPK family has been documented to be synthetically lethal with deregulated MYC activity. The conceptual framework developed by Kfoury et al. seems insufficiently insightful. Specifically, the authors also show that loss of MYC function also induced death of melanoma cells in a fashion that could be protected by activating AMPK; this converse phenomenon, however, does not shed any conceptual light on why AMPK is required when MYC is high. This work could provide additional mechanistic insight by further delineating how AMPK attenuates MYC-induced cell death. Here, the potential concept is that AMPK provides a negative feedback loop for deregulated MYC-induced biosynthesis, whereby continual consumption of energy induced by MYC for biosynthesis would be counterbalanced by AMPK that provides continuous breaks on ATP consumption and allows the system to take up nutrients for ATP production as well as biosynthesis in a balanced way. As such, the need for AMPK could be alleviated partly by slowing down MYC-induce biosynthesis - via, for example, inhibiting Pol III activity, which is essential for tRNA and ribosome biogenesis, inhibiting protein synthesis (cycloheximide), or inhibiting nucleic acid synthesis (leflunomide, or IMPDH inhibition). Direct measurements of AMP, ADP, and ATP or metabolomic analysis (which may be well beyond the scope of this study) could provide significant mechanistic insights as well. Without such mechanistic insight, the current version of the manuscript appears quite descriptive and corroborative of earlier studies implicating MYC downstream of RAS and the essentiality of AMPK for MYC-induced tumorigenesis.

Minor: The authors are encouraged to update their citations particularly of review articles on MYC (several cited in the manuscript are quite outdated).

Referee #2:

Using different mouse models Kfoury et al demonstrate that c-Myc is important for melanoma development and progression downstream of mutant NRas. By looking at the potential of tumour initiating cells the authors show that Myc positive mouse melanoma cells are more likely to give rise to metastasis than Myc negative cells. The authors show in in vitro functional studies that Apmk is downstream of Myc and enhances survival and growth in Myc positive cells. Furthermore the authors demonstrate that high MYC expression correlates with poor survival in the TCGA melanoma cohort. In general the work is well presented and provides strong supporting evidence for the role of Myc in Ras driven melanoma in the mouse models. The link of MYC-AMPK and its role in human melanoma is less substantiated.

Major points

1. Re Figure 1. The reduction in residual melanocytes in the Myc depleted mice is not a trivial issue, as this will directly impact on the onset of tumour growth and the ability to form melanomas. Do these mice develop benign nevi? If not this might suggest a general melanocyte depletion/reduction phenotype rather than a phenotype of lack of transformation/initiation. To at least consider the possibility the number of residual skin melanocytes in wt and Myc depleted mice should be

quantified and shown.

2. Re Figure 3. Figure 3C assesses the role of Myc in tumour maintenance by quantifying the in vivo growth of the different Myc cell-lines. The control for 4OHT treated mM1 cre-ERT-IRES-GFP cells (compared to mM1) is shown in Figure EV3E, where 1x10˜5 cells had been injected and mice have been treated with 4OHT at day 10 with a volume of app 20mm˜3. The 'tumour maintenance' experiment however uses 10˜6 cells whereby tumours reach a size of app 150mm˜3 within 4 days when the mice are treated with 4OHT. 4 days gives hardly enough time for a 'proper' tumour to develop through growth. Rather this experiment appears to look at cell death as a consequence of Myc depletion in cells expressing CRE within a cell 'aggregate' in vivo, and when the 4OHT treatment is stopped the cells that did not express high levels of CRE in the firs place and therefore did not lose Myc take over growth. The experiment needs to be repeated with 10˜5 cells, whereby tumours can properly establish and probably will represent a more realistic (possibly heterogeneous) expression of Myc. At the time of 4OHT treatment. The depletion of Myc will show whether Myc was and is the driver of tumour growth and is required for tumour maintenance.

3. For the tumour maintenance experiment histology for KI67 and an apoptosis marker as well as Myc itself should be performed in order to assess the extent of Myc loss and the consequences for proliferation and survival.

4. In Figure 5 results of mM1 cells need to be added (where data not shown is mentioned) to be able to compare these against mM2 cells to insure the effect is Myc expression dependent.

5. I could not find any demonstration that the chosen concentration (and timing) for the 991 AMPK activator actually leads to a significant activation of AMPK, which should be assessed by the downstream factors that have been analysed in Figure 5A and B. This is particularly important to show, as according to Figure 5A the reduction in AMPK expression after Myc depletion is quite severe and simply activation of the residual amount might not be enough to fully restore the untreated situation, and hence the effects of 991 might be 'off-target'. Otherwise overexpression of active AMPK might be another approach.

6. Histology for AMPK or likewise in the mouse control and Myc depleted tumours would be very supportive of the proposed mechanism

7. Because the authors aim to link their findings in mouse cells to human melanoma the relevance of the MYC-AMPK-ROS connection needs to be shown in human melanoma cell lines, best a couple of primary and metastatic lines from the panel that is described in Figure 7A. This is crucial as so far the presented human data are only correlative. Also, does AMPK expression follow MYC expression in the human melanoma cell lines?

8. Is high/low AMPK expression correlated with a similar effect on patient survival? How does AMPK expression link to myc expression in histology in the tumours of patients?

9. Generally the loading control Actin used in some of the blots is highly over exposed and in places impossible to easily assess the expression changes 3A/4E/5ABCE/6B these should be replaced with lower exposures and where necessary the whole blots may need replacing (5A in particular). In EV5 the Actin blots look like possible duplications; source data/ original scans for all blots should be provided.

Minor points

Why have immune incompetent mice been used instead of an allograft model for re-implanting Myc GFP cells?

Highlighting the genes of interest in figure 4D, and adding the human gene names would be helpful.

Referee #3:

The study by Kfoury et al shows a genetic requirement for MYC during formation of melanoma in a GEMM model driven by melanocyte-specific expression of mutant NRas on a Cdkn2a null background. Although the requirement for MYC in RAS-driven cancers has been shown previously, this is the first demonstration of such a requirement specifically in melanoma. The authors go on to show that melanoma cells with high MYC expression become dependent upon AMPK for survival and show that depletion or inhibition of AMPK results in ROS-dependent melanoma cell death. This is an area of some controversy as AMPK has previously been shown to play both pro- and anti-cancer roles in different contexts. As such, these results are interesting and the study does advance our understanding of these complex interactions. The manuscript is well written, clear and the experiments are by-and-large well conducted. I do however have some concerns regarding mechanism of action and some of the experiments require additional controls

Major points:
1) Although the genetic demonstration that MYC is required for melanoma formation is important, a more clinically relevant question is whether pharmacological targeting of MYC expression in established tumours has any therapeutic benefit. Thus, does JQ1 induce apoptosis or otherwise show therapeutic benefit in either of the in vivo melanoma models? Does JQ1 influence expression of the AMPK subunits in melanoma cells?

2) Does MYC transcriptionally regulate expression of specific AMPK subunits in the melanoma cells? This has not been reported in other cell types and would be a novel observation. What is the status of NUAK1 (ARK5) upon MYC depletion?

3) In Fig.5, although the dorsomorphin and individual depletion of Prkaa1 and Prkab2 do show good agreement, dorsomorphin, as correctly pointed out by the authors, is not a particularly selective AMPK inhibitor and only 1 siRNA is used for each of the subunits. The authors should be able to reproduce these data with a second siRNA for each Prkaa1 and Prkab2. Does depletion of Prkab1 have any effect on viability?

4) The protective effects of the AMPK activator 991 are quite profound but it is unclear how the activator can be so effective when the levels of AMPK are so reduced. Does 991 stabilise AMPK? The authors should show the effects of 991 treatment on AMPK target phosphorylation in the presence and absence of MYC.

5) What is the status of AMPK subunit expression in the patient-derived melanoma cell lines? Is there any correlation with patient survival?

Minor points:
1) The gene expression analysis (Fig. 4) appears to have been conducted on a mixture of primary and metastatic samples (and low numbers of each). Given that MYC levels are clearly higher in the metastases, and given the profound differences in the local microenvironment in each tissue, it is impossible to tell which of these factors is driving the observed gene expression differences. The authors should increase the sample size here and deconvolute primary samples from metastases.

2) What is the status of N-Myc in the c-Myc-deleted melanoma model? The KM plot stops at 6 months by which time none of the floxed MYC mice develop melanoma - is it still appropriate to say that that these mice "never develop melanoma" as claimed?

3) In the section dealing with the levels of MYC-GFP expression in primary and metastatic sites, it would be better to refer to MycHigh and MycLow, rather than Myc+ and Myc-, for clarity.

4) The authors may wish to note that depletion of AMPKα1 was previously shown to be synthetic lethal with MYC overexpression in the study by Liu, Ulbrich et al (which they do cite).

1st Revision - authors' response 20 December 2017

Point by point response to referees comments and summary of revisions to manuscript:

Referee #1:

The manuscript by Kfoury et al. reports a role for MYC in NRAS-driven Ink4a null melanoma, particularly regarding aggressive and metastatic disease that is dependent on AMPK activity. The authors used genetically engineered mouse models and cells to glean the role of MYC, which is known to play a critical role in cellular growth metabolism and also previously shown to underpin the tumorigenic potential of KRAS (hence RAS/RAF/MEK pathways) in a transgenic lung cancer model. As such, this specific role of MYC downstream of RAS is not a new concept.

We agree that Kras and c-myc have previously been shown to cooperate in other tumor models such as in a model of $Kras^{G12D}$-driven lung adenomas, where additional c-Myc activation induces a fast transition of adenomas into a highly proliferative and invasive adenocarcinoma. Although, the concept per se might be known and therefore not novel, the precise mechanisms underlying this transition are not known and may differ from tumor type to tumor type. In this context, it is remarkable to note that an interesting paper was just published in the last issue of Cell by the group of Gerard Evan showing that Myc cooperates with Ras by programming the tumor microenvironment, which becomes tumor suppressive (Kortlever et al. 2017 Cell 171, 1301-1315.) The mechanisms by which the Myc-Ras connection might promote tumorigensis might differ from tumor type to tumor type. We think that the role of c-Myc in melanoma is under-investigated and that our results provide new interesting insights.

However, the authors did probe the role of AMPK and found that high MYC-mediated melanomas are dependent on AMPK activity such that loss of function of AMPK resulted in cell death, presumably due to excessive redox stress.The source of redox stress, however, is undefined. Here, it should be noted that AMPK family has been documented to be synthetically lethal with deregulated MYC activity.

We thank the referee for pointing this out. Indeed the study by Liu, Ulbrich et al published in *Nature* **483**, 608–612 (29 March 2012) showed that ARK5 can be synthetic lethal with deregulated c-Myc expression in UOS2 cells and hepatocellular carcinoma cell lines (please see also referee 3 point 2). We therefore investigated whether this mechanism might also be conserved in melanoma. However, we could not detect any ARK5 expression in our melanoma cell lines, indicating that ARK5 in this tumor context is not linked to Myc. See also point 2 of referee 3 and attached Rebuttal Figure 4.

The conceptual framework developed by Kfoury et al. seems insufficiently insightful.

We regret that the conceptual framework of our first version of the manuscript was conceived as insufficiently insightful. But we hope with the additional data provided our study has sufficiently improved so that the new revised manuscript can now be considered for publication in The EMBO Journal.

Specifically, the authors also show that loss of MYC function also induced death of melanoma cells in a fashion that could be protected by activating AMPK; this converse phenomenon, however, does not shed any conceptual light on why AMPK is required when MYC is high. This work could provide additional mechanistic insight by further delineating how AMPK attenuates MYC-induced cell death. Here, the potential concept is that AMPK provides a negative feedback loop for deregulated MYC-induced biosynthesis, whereby continual consumption of energy induced by MYC for biosynthesis would be counterbalanced by AMPK that provides continuous breaks on ATP consumption and allows the system to take up nutrients for ATP production as well as biosynthesis in a balanced way. As such, the need for AMPK could be alleviated partly by slowing down MYC-induce biosynthesis - via, for example, inhibiting Pol III activity, which is essential for tRNA and ribosome biogenesis, inhibiting protein synthesis (cycloheximide), or inhibiting nucleic acid synthesis (leflunomide, or IMPDH inhibition). Direct measurements of AMP, ADP, and ATP or metabolomic analysis (which may be well beyond the scope of this study) could provide significant mechanistic insights as well. Without such mechanistic insight, the current version of the manuscript appears quite descriptive and corroborative of earlier studies implicating MYC downstream of RAS and the essentiality of AMPK for MYC-induced tumorigenesis.

We thank the reviewer for these constructive suggestions that helped us to improve our manuscript and obtain further mechanistic insight.
We performed the following experiments as suggested. To partially alleviate the need for AMPK, mM1 and mM2 melanoma cells were treated with the AMPK inhibitor Dorsomorphin in presence or absence of a Pol III inhibitor (Merck 557403). Inhibition of Pol III will slow down Myc-induced t-RNA and ribosome biogenesis. Dorsomorphin-induced apoptosis as a consequence of AMPK inhibition was indeed significantly reduced in both mM1 and mM2 melanoma cell lines indicating that slowing down t-RNA and ribosomal biogenesis can in part alleviate the requirement for AMPK. These new data are now shown in Fig 7D, mentioned in the manuscript on page 13 & 14 and are in agreement with AMPK providing a negative feedback loop for deregulated MYC-induced biosynthesis.

Moreover, we performed additional experiments with other inhibitors as suggested. Vehicle or Dorsomorphin treated mM1 and mM2 cells were grown in the presence or absence of leflunomide or mycophenolic acid (inhibits nucleic acid synthesis), or cycloheximide (inhibits protein synthesis). Although we carefully titrated the concentrations used for individual inhibitors used they all induced a significant level of apoptosis already in vehicle treated mM1 and mM2 cells within the experimental setting of 48h. As an example, results are shown for leflunomide and mycophenolic acid for the referee's perusal. These results were not included in our manuscript as they are not conclusive (Rebuttal Figure 1).

We concur with the reviewer on his point of view that a complete metabolomic analysis is beyond the scope of our manuscript. We nevertheless monitored cellular energy status by measuring adenine nucleotide levels (ATP, ADP and AMP) in control and Myc depleted mM2 cells using ultra-high performance liquid chromatography mass spectrometry (Pluskal, T et al. *Mol. Biosyst.* 2009, *6*, 182–198) as suggested. ATP, ADP and AMP measurements were normalized to either protein content or total amino acid content. The outcome was the same, independent of the way of normalization. Genetic c-Myc depletion caused a robust and significant decrease in ATP and ADP levels, while AMP levels were not significantly altered, indicating that c-Myc is an important driving force for ATP production. Consequences of c-Myc depletion were monitored assessing the phosphorylation status of known AMPK targets.
As an additional benchmark we also measured the nucleotide changes in control versus mM2 knocked-down for both AMPKα1. The efficency of the AMPKα1 knockdown and the consequences on their target proteins were verifed by Western blot analysis. Consistent with the results of c-Myc depletion, knockdown of AMPKα1 resulted also in significantly decreased ATP and ADP levels (Fig EV5). The reduction in ATP and ADP levels in AMPK depleted cells was less pronounced compared to c-Myc deficient cells, which is consistent with the possibility that c-Myc exerts its important function in ATP biosynthesis not exclusively through AMPK.

These results are shown as Fig EV5 and mentioned in the revised manuscript on page 14.

Minor: The authors are encouraged to update their citations particularly of review articles on MYC (several cited in the manuscript are quite outdated).

We completely agree and updated our citations particularly the review articles on Myc.

Referee #2:

Using different mouse models Kfoury et al demonstrate that c-Myc is important for melanoma development and progression downstream of mutant NRas. By looking at the potential of tumour initiating cells the authors show that Myc positive mouse melanoma cells are more likely to give rise to metastasis than Myc negative cells. The authors show in in vitro functional studies that Apmk is downstream of Myc and enhances survival and growth in Myc positive cells. Furthermore the authors demonstrate that high MYC expression correlates with poor survival in the TCGA melanoma cohort. In general the work is well presented and provides strong supporting evidence for the role of Myc in Ras driven melanoma in the mouse models. The link of MYC-AMPK and its role in human melanoma is less substantiated.

We want to thank this reviewer for his/her positive comments and constructive criticism. We also agree with the remark that the link of Myc-AMPK in human melanoma was underdeveloped in our first version of the manuscript, which has now been addressed as suggested.

Major points

1. Figure 1. The reduction in residual melanocytes in the Myc depleted mice is not a trivial issue, as this will directly impact on the onset of tumour growth and the ability to form melanomas. Do these mice develop benign nevi? If not this might suggest a general melanocyte depletion/reduction phenotype rather than a phenotype of lack of transformation/initiation. To at least consider the possibility the number of residual skin melanocytes in wt and Myc depleted mice should be quantified and shown.

We agree and indeed Tyr-Cre mediated inactivation of c-Myc in Tyr::NrasINK4a$^{-/-}$ mice results in a reduction of melanocytes which is in agreement with previous studies by Pshenichnaya et al 2012 as mentioned in our first manuscript (page 6). Although the mice have reduced melanocyte numbers, they never progress to melanoma. To quantitatively compare melanocyte numbers, we first tried to perform immuno-staining using a new commercially available antibody against the melanocyte marker dopachrome tautomerase (Trp2 sc-10452, Santa Cruz Biotechnology, Santa Cruz, CA). The previously used homemade antibody (kind gift from H. Hearing, NIH, Bethesda) was consumed. Unfortunately, the commercial Trp2 antibody from Santa Cruz resulted only in unspecific staining. We therefore opted to perform Fontana Masson staining, which stains melanin, on skin sections derived from Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{lox/lox}$, Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$ and C57BL/6 WT animals. Skin samples obtained

from *NSG* mice lacking melanocytes have been used as negative control (Fig 1B). While mice developing melanoma show abundant melanin positive cells, c-Myc mutant mice reveal a strong reduction but residual melanin positive cells are visible. We also quantified the overall melanin content in the skin of these animals. Hair was removed to avoid contaminating melanin. Although the melanin content of Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$ mice was 15.9-fold reduced compared to Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{lox/lox}$ it was comparable to C57BL/6 WT mice. The melanin content measurements are in agreement and correlate with the Fontana Masson staining. Taken together these results suggest that Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$ do have residual melanocytes that do not develop into melanoma.

These new results are now provided as revised Fig 1 and mentioned in the text on page 6 of the revised manuscript. We agree with referee that the inability of Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$ mice to develop melanoma is likely a combination of both, a reduction of melanocytes and thus potential loss of tumor initiating cells and the absence of c-Myc, which does not allow remaining melanocytes to develop into melanomas.

2. Re Figure 3. Figure 3C assesses the role of Myc in tumour maintenance by quantifying the in vivo growth of the different Myc cell-lines. The control for 4OHT treated mM1 cre-ERT-IRES-GFP cells (compared to mM1) is shown in Figure EV3E, where 1x10˘5 cells had been injected and mice have been treated with 4OHT at day 10 with a volume of app 20mm˘3. The 'tumour maintenance' experiment however uses 10˘6 cells whereby tumours reach a size of app 150mm˘3 within 4 days when the mice are treated with 4OHT. 4 days gives hardly enough time for a 'proper' tumour to develop through growth. Rather this experiment appears to look at cell death as a consequence of Myc depletion in cells expressing CRE within a cell 'aggregate' in vivo, and when the 4OHT treatment is stopped the cells that did not express high levels of CRE in the firs place and therefore did not lose Myc take over growth. The experiment needs to be repeated with 10˘5 cells, whereby tumours can properly establish and probably will represent a more realistic (possibly heterogeneous) expression of Myc. At the time of 4OHT treatment. The depletion of Myc will show whether Myc was and is the driver of tumour growth and is required for tumour maintenance.

We agree and performed new tumor maintenance experiments as suggested. Please see also point 1 of referee 3 who suggested to pharmacologically target Myc expression in established tumors to evaluate a potential therapeutic benefit. Therefore, we decided to pharmacologically block Myc in new tumor maintenance experiments. As suggested, 1x10^5 mM1 melanoma cells were injected s.c. into Rag2γc mice (n=11/per group). Once the tumor size reached

approximately 100mm^3, mice were split into two groups and treated either with (+) or (-)JQ1 (50 mg/kg/day). (+)JQ1 treatment resulted in a clear and significant reduction of tumor growth compared to the (-)JQ1 treated cohort. Tumors were harvested at two different time points (5 and 9 days) post JQ1 treatment for histological analysis as suggested under point 3. Comparable experiments were performed with another independent melanoma cell line (mM3 generated in our lab from Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{wt/wt}$) to exclude the possibility that the results might only be specific to mM1 melanoma cells. For mM3 (-) and (+)JQ1 treatment was initiated once tumor sizes were approximately 50mm^3.
Thus, a new Fig 4 replaces now previous Fig 3C. The results of mM3 are show as Fig EV3E. The results are described on page 9 of the revised manuscript.

3. For the tumour maintenance experiment histology for KI67 and an apoptosis marker as well as Myc itself should be performed in order to assess the extent of Myc loss and the consequences for proliferation and survival.

We thank the reviewer for this suggestion and performed histological analysis for, c-Myc, Ki67, cleaved caspase 3 and p-AMPK (as suggested under point 6). Tumors were harvested at 5 and 9 days post JQ treatment for histological. (+)JQ1 treated animals show reduced staining for c-Myc and Ki67, compared to tumors of the (-)JQ1 treated animals. Quantification of Ki67 stained tumors indicates a reduction of 57% of Ki67 positve cells in (+)JQ1 treated tumors compared to (-) JQ1. Cleaved Caspase 3 staining was comparable between the two cohorts indicating that *in vivo* (+)JQ1 mediated tumor growth retardation is mostly due to inhibition of proliferation. Staining's were similar for both time points investigated. These new data are now shown as Fig 4B and mentioned on page 9 of our revised manuscript.

4. In Figure 5 results of mM1 cells need to be added (where data not shown is mentioned) to be able to compare these against mM2 cells to insure the effect is Myc expression dependent.

We agree and as suggested results of mM1 cells have now been added and are shown as modified Fig EV4A, B, C and mentioned in the text of the revised manuscript on page 12.

5. I could not find any demonstration that the chosen concentration (and timing) for the 991 AMPK activator actually leads to a significant activation of AMPK, which should be assessed by the downstream factors that have been analysed in Figure 5A and B. This is particularly important to show, as according to Figure 5A the reduction in AMPK expression after Myc depletion is quite severe and simply

activation of the residual amount might not be enough to fully restore the untreated situation, and hence the effects of 991 might be 'off-target'. Otherwise overexpression of active AMPK might be another approach.

We added now additional Western blot analysis (Fig 6A and E, previously Fig 5) to show the activation of AMPK downstream targets through 991 in MEFs (Fig 6A) and mM2 cells (Fig 6E).
It is important to note that the Western blot analysis in Fig 6A showing a severe reduction of AMPK has been done 48h after c-Myc inactivation. In contrast, in Fig 6E the 991 AMPK activator was added simultaneously with 4-OHT. Thus, 991 was present from the start of the experiment, when AMPK levels were normal. Fresh 991 was added every 24hrs of the experiment to activate the remaining amount of AMPK. In addition, we repeated the experiment shown in Fig 6E but instead of using 991 we expressed a C-terminally truncated constitutive active form of AMPK (AMPK CA, Crute et al. 1998, JBC, Vol253, No52, Dec 25, pp35347-35354) and obtained essentially the same results as shown in Fig 6E. Expression of a dominant active form of AMPK in mM2 cells was able to protect melanoma cells against c-Myc depletion induced apoptosis. Thus, we used two independent methodologies to show the protective effect of activating AMPK in a situation of c-Myc depletion. These new results are now shown in Fig EV4E and mentioned on page 12 of our revised manuscript.

6. Histology for AMPK or likewise in the mouse control and Myc depleted tumours would be very supportive of the proposed mechanism

Histology for AMPK staining was performed using the phospho-specific anti-AMPK antibody recognizing the active forms of AMPKα1 and α2. The specificity of p-AMPK staining was first confirmed by tretating MEFs and AMPKα1/α2 DKO MEFs as negative controls with the AMPK activator AICAR (Rebuttal Figure 2), before staining tumor sections derived from the tumor maintenance experiment shown in Fig 4A. As shown in Fig 4B (+)JQ1 but not (-) JQ1 treated tumors showed reduced c-Myc, Ki67 and p-AMPK staining (Fig EV 4F). These results are now mentioned on page 10 of our revised manuscript.
The same antibody was used on sections from clinical specimens of human melanoma patients, which show strong p-AMPK staining in metastatic samples, while primary melanoma samples were largely negative for p-AMPK. Fig 9A, now mentioned on page 15 and 16 of our revised manuscript.

7. Because the authors aim to link their findings in mouse cells to human melanoma the relevance of the MYC-AMPK-ROS connection needs to be shown in human melanoma cell lines, best a couple of primary and metastatic lines from

the panel that is described in Figure 7A. This is crucial as so far the presented human data are only correlative. Also, does AMPK expression follow MYC expression in the human melanoma cell lines?

We agree and thank the reviewer for this comment. As suggested, we investigated the consequences of siRNA-mediated knockdown of AMPKα1 in a series of human cell lines shown in our previous Fig 7A, which is now Fig 8. Following cell lines were used: LAU-T921 primary melanoma, low C-MYC expression, LAU-Me 252, metastatic melanoma line with low C-MYC expression, LAU-Me275 metastatic melanoma line with high C-MYC expression and LAU-T333 metastatic melanoma with high C-MYC expression. Knockdown of AMPKα1 in the primary (LAU-T921) or metastatic cell lines (LAU-Me 252) with low C-MYC expression did not result in induction of a apoptosis or ROS production, in contrast to both metastatic cell lines (LAU-Me275, LAU-T333) with high C-MYC expression, which both showed a very significant increase in apoptotic cells and ROS production. These results are consistent with our melanoma mouse data and suggest that survival of the investigated metastasis-derived human melanoma cell lines with high C-MYC expression are also dependent on AMPK activity to suppress oxidative stress. These data are now shown as Fig 8C,D and mentioned on page 15 of our revised manuscript. .

8. Is high/low AMPK expression correlated with a similar effect on patient survival? How does AMPK expression link to myc expression in histology in the tumours of patients?

To test whether AMPK expression levels might correlate with poor prognosis we analyzed TCGA database case sets and correlated survival of melanoma patients based on AMPKα protein expression of 192 patients, which were classified into high and low expressing cohorts. The high expression cohort corresponds to the 33% of patients expressing highest AMPKα protein levels whereas the low cohort includes the 33% of patients with the lowest expression. Although not significant because AMPKα high and low Kaplan Meyer curves cross each other in particular in the first two years after diagnosis, patients that survive beyond three years of diagnosis show a clear trend with a median survival of 1871 days for patients with high AMPKα protein levels compared to 4000 days of patients expressing low AMPKα protein levels at diagnosis. Thus, the reduction in median survival time (MST) for AMPKα high versus AMPKα low expressing melanoma patients is 5.8 years. This analysis is shown as Fig 9E and mentioned on page 17 of our revised manuscript.

9. Generally the loading control Actin used in some of the blots is highly over

exposed and in places impossible to easily assess the expression changes 3A/4E/5ABCE/6B these should be replaced with lower exposures and where necessary the whole blots may need replacing (5A in particular). In EV5 the Actin blots look like possible duplications; source data/ original scans for all blots should be provided.

Wherever possible we now show lower exposures of our actin loading controls. Fig 6A (previously Fig 5) has been expanded also in request of referee 3 (point 4), it now also shows Western blot analysis of all AMPK subunits in mM1 and mM2 cells treated either with (-) or (+)JQ1. Moreover, we show new Western blot analysis of AMPK downstream target proteins of 991 treated (24h) MEFs (Fig 6A). This serves to show that the small molecule 991 indeed enhances AMPK activity as previously published by Bultot et al. 2016 (doi: 10.1152/ajpendo.00237.2016).
Thank you for pointing out the duplication of the actin loading control in our original Fig EV5. This is indeed a mistake. Since AMPKβ2 and AMPKα1 both were probed on the same blot thus only one loading control should have been shown. The Western blot figure should have been mounted differently. The corrected Figure is now shown as Fig EV4D. Original scans of blots will be provided.

Minor points

10. Why have immune incompetent mice been used instead of an allograft model for re-implanting Myc GFP cells?

We used immune compromised mice as recipients in our transplant settings since Myc GFP cells would not engraft and grow in an allograft transplant model. In general tumor cells across allogeneic borders are usually rejected by the immune system of the host.

11. Highlighting the genes of interest in figure 4D, and adding the human gene names would be helpful.

Thank you for this suggestion. We have now highlighted the genes of interest and added the human gene names in brackets.

Referee #3:

The study by Kfoury et al shows a genetic requirement for MYC during formation of melanoma in a GEMM model driven by melanocyte-specific expression of mutant NRas on a Cdkn2a null background. Although the requirement for MYC in

RAS-driven cancers has been shown previously, this is the first demonstration of such a requirement specifically in melanoma. The authors go on to show that melanoma cells with high MYC expression become dependent upon AMPK for survival and show that depletion or inhibition of AMPK results in ROS-dependent melanoma cell death. This is an area of some controversy as AMPK has previously been shown to play both pro- and anti-cancer roles in different contexts. As such, these results are interesting and the study does advance our understanding of these complex interactions. The manuscript is well written, clear and the experiments are by-and-large well conducted. I do however have some concerns regarding mechanism of action and some of the experiments require additional controls

We thank this reviewer for his/her positive comments and constructive criticism.

Major points:

1) Although the genetic demonstration that MYC is required for melanoma formation is important, a more clinically relevant question is whether pharmacological targeting of MYC expression in established tumours has any therapeutic benefit. Thus, does JQ1 induce apoptosis or otherwise show therapeutic benefit in either of the in vivo melanoma models? Does JQ1 influence expression of the AMPK subunits in melanoma cells?

We agree that this is an important question and thank the reviewer for the suggestion of this experiment. Please see also comment of referee 2 point 2 and our reply.

To address this point $1x10^5$ mM1 melanoma cells were injected s.c. into *Rag2γc* mice (n=11/per group). Once tumor size reached approximately $100mm^3$, animals were split into two cohorts and treated either with (+) or (-)JQ1 (50 mg/kg/day). (+)JQ1 treatment resulted in a clear and significant reduction of tumor growth compared to the (-)JQ1 treated cohort. Tumors were harvested at two different time points (5 and 9 days) post JQ1 treatment for histological analysis as suggested by reviewer 2 point 3. Two independent experiments were performed. Identical experiments were performed with another independent melanoma cell line (mM3 - generated in our lab from a Tyr::$Nras^{Q61K}$INK4a$^{-/-}$c-$Myc^{wt/wt}$ animal) to exclude the possibility that the results might only be specific to mM1 melanoma cells. Taken together our results show that (+)JQ1 treatment has a clear therapeutic benefit.
A new Fig 4 replaced Fig 3 C. The results of mM3 are show as Fig EV3E and are described on page 9 of our revised manuscript.

2) Does MYC transcriptionally regulate expression of specific AMPK subunits

in the melanoma cells? This has not been reported in other cell types and would be a novel observation.

We agree that this is an interesting question and as pointed out by the referee it is currently unknown whether AMPK subunits are transcriptionally regulated by c-Myc or whether this is an indirect process. To address this question we focused on the Prkab2 gene, which according to our RNA seq data showed the most significant change among the AMPK subunits in RNA expression between c-Myc^{lo} and c-My^{hi} melanoma samples analyzed. The following experiments were performed. We identified a potential c-Myc binding site within the 2kb-promoter region of the Prkab2 gene. This Prkab2 promoter region was cloned upstream of a luciferase promoter construct, which was transiently transfected into HeLa cells, HeLa cells expressing an inducible Myc-ER construct and HeLa cells in which c-Myc was depleted. In none of the conditions tested a convincing change in luciferase activity could be observed (see Rebuttal Fig 3A,B) indicating that this promoter region is not likely to be directly regulated by c-Myc.
However, these results do not exclude the possibility that c-Myc could still regulate Prkab2 transcription directly through other may be more distant regulatory elements. To further test this possibility, we introduced a doxycycline regulated c-Myc transgene in mM2 melanoma cells and investigated mRNA expression 6 and 10h after doxycyclin-induced expression of c-Myc. No significant change in the expression of the Prkab2 mRNA under the different conditions tested was observed (Rebuttal Figure 3C). Further experiments within the given time frame to address this question were not possible. Based on our current data we cannot conclude that c-Myc is directly regulating the expression Prkab2. It is therefore possible that c-Myc may regulate the expression of Prkab2 and possibly other subunits through indirect mechanisms. In this context it is interesting to note that Prkab2 was identified as an indirect Myc target in a study by Kress et al investigating Myc-dependent transcriptional programs in ongogene-addicted liver tumors (Kress et al. Cancer Res. 2016 Jun 15;76(12):3463-72. doi: 10.1158/0008-5472.CAN-16-0316. Epub 2016 Apr 13., Supplementary Table S1.)

What is the status of NUAK1 (ARK5) upon MYC depletion?

We thank the reviewer for this question. This is an important point because ARK5 was previously shown to be synthetic lethal with deregulated c-Myc expression in UOS2 cells and hepatocellular carcinoma. To address this question we performed Western blot analysis for ARK5 in control and c-Myc siRNA treated mM1 cells as well as EtOH and 4-OHT treated mM2 cells. Mouse testis and brain were used as positive control. While AKR5 is expressed in brain and very strongly in testis, we could not detect any AKR5 expression in mM1 or mM2 cells independent of the c-Myc status suggesting that AKR5 in our murine melanoma

model system does seem not to be relevant or linked to Myc as has been previously been shown in the Liu et al paper (Nature 29 March 2012, doi: 10.1038/nature10927) for U2OS cells and hepatocellular carcinoma. Since we opted not to show the data in the paper, we make it available here for the perusal of the referee (Rebuttal Fig 4).

3) In Fig.5, although the dorsomorphin and individual depletion of Prkaa1 and Prkab2 do show good agreement, dorsomorphin, as correctly pointed out by the authors, is not a particularly selective AMPK inhibitor and only 1 siRNA is used for each of the subunits. The authors should be able to reproduce these data with a second siRNA for each Prkaa1 and Prkab2.

This seems to be a misunderstanding. In the knockdown experiments for Prkaa1 and Prakb2 of our original manuscript we used a smart pool from Qiagen consisting of four different siRNAs as was mentioned in materials and methods. We apologize if we have not been precise enough in our first manuscript. This has now been corrected. Moreover, we ordered individual siRNAs from the respective smart pool for both Prkaa1 (AMPKα1) and Prkab2 (AMPKβ2) and tested those individually on mM1 and mM2 melanoma cells. All four individually tested siRNAs induced cell death and were verified by Western blot analysis. The results are shown as Rebuttal Figure 5 for the referee's perusal.

Does depletion of Prkab1 have any effect on viability?

To address this question we performed siRNA-mediated knockdown for Prkab1 (AMPKβ1) in mM1 and mM2 melanoma cells. We again used a smart pool siRNA (Qiagen) for Prkab1 consisting of a pool of four individual siRNAs. The knockdown of Prkab1 did not show any significant induction of apoptosis in mM2 cells at two independent time points of analysis (48 and 72h). Similarly, knockdown of Prkab1in mM1 cells 48h post transfection did not induce apoptosis significantly. At the later time point of analysis (72hrs), a mild induction of apoptosis was noted (20 versus 35%). Taken together, these results indicate that knockdown of Prkab1 does not have a major impact on cell viability. These results are shown as Rebuttal Figure 6 for the referee's perusal.

4) The protective effects of the AMPK activator 991 are quite profound but it is unclear how the activator can be so effective when the levels of AMPK are so reduced. Does 991 stabilise AMPK? The authors should show the effects of 991 treatment on AMPK target phosphorylation in the presence and absence of MYC.

We agree that this might not be easy to understand. Please see also point 5 of referee 2 who commented on the same issue.
It is important to note that Western blot analysis in Fig 6A showing a severe reduction of AMPK has been performed 48hrs after c-Myc gene inactivation. In contrast, in Fig 6E 991 was added simultaneously with 4-OHT. Thus, the AMPK activator 991 was present from the initial phase of the experiment, when AMPK levels were normal. Fresh 991 was added every 24hrs to the experiment to ensure full activation of the remaining amount of AMPK. In addition, we repeated the experiment shown in Fig 6E but instead of using 991 we expressed a C-terminally truncated dominant active form of AMPKα1 (Crute et al. 1998, JBC, Vol253, No52, Dec 25, pp35347-35354) and obtained essentially the same results as shown in Fig 6E. Expression of a dominant active form of AMPK in mM2 cells was able to protect melanoma cells against c-Myc depletion induced apoptosis. Thus, we used two independent methodologies to show the protective effect of activating AMPK in a situation of c-Myc depletion. These new results are now shown in Fig EV4E and mentioned on page 12 of our revised manuscript.

5) What is the status of AMPK subunit expression in the patient-derived melanoma cell lines? Is there any correlation with patient survival?

To assess the status of AMPK subunit expression we used four cell lines from our original Fig 8A, now 9A. The panel of following cell lines was used: LAU-T921 primary melanoma, low C-MYC expression, LAU-Me 252, metastatic melanoma line with low C-MYC expression, LAU-Me275 metastatic melanoma line with high C-MYC expression and LAU-T333A metastatic melanoma with high C-MYC expression. These are the same cell lines that were used to address consequences of AMPKα1 knockdown (please see also Referee 2, point 7 and 8).
All tested cell lines express all AMPK subunits, though to variable levels, with the exception AMPKα2, which is not detectable in LAU-T333A. These results are now shown as (Fig 8B) and mentioned on page 15 of our revised manuscript.

To test whether AMPK expression levels might correlate with poor prognosis we analyzed TCGA database case sets and correlated survival of melanoma patients based on AMPKα protein expression of 192 patients, which were classified into high and low expressing cohorts. The high expression cohort corresponds to the 33% of patients expressing highest AMPKα protein levels whereas the low cohort includes the 33% of patients with the lowest expression. Although not significant because AMPKα high and low Kaplan Meyer curves cross each other in particular in the first two years after diagnosis, patients that survive beyond three years of diagnosis show a clear trend with a median survival of 1871 days for patients with high AMPKα protein levels compared to

4000 days of patients expressing low AMPKα protein levels at diagnosis. Thus the reduction in median survival time (MST) for AMPK high versus AMPK low expressing melanoma patients is 5.8 years. This analysis is shown as Fig 9E and mentioned on page 17 of our revised manuscript.

Minor points:

1) The gene expression analysis (Fig. 4) appears to have been conducted on a mixture of primary and metastatic samples (and low numbers of each). Given that MYC levels are clearly higher in the metastases, and given the profound differences in the local microenvironment in each tissue, it is impossible to tell which of these factors is driving the observed gene expression differences. The authors should increase the sample size here and deconvolute primary samples from metastases.

Although listed under minor point this is not trivial, as it takes 7-8 months for these melanoma mice to develop metastasis, which is beyond the time the editors allocated for our revisions. We therefore decided to add one more LN sample, which was previously done, deconvolve the analysis and compare gene expression of primary melanoma samples of MYC low versus high and LN metastasis of MYC low versus high. The results are basically the same to the previous analysis and also after deconvolution did not change.

2) What is the status of N-Myc in the c-Myc-deleted melanoma model?

To address this important point we performed Western blot analysis for N-Myc in control and c-Myc depleted mM1 and mM2 cells. The SK-N-BE(2)-C neuroblastoma cell line was used as positive control. While N-Myc was clearly detected in protein samples prepared from the neuroblastoma cell line it was not expressed in our mM1 and mM2 melanoma cells. The results are shown as Rebuttal Fig 7 for the reviewer's perusal.

The KM plot stops at 6 months by which time none of the floxed MYC mice develop melanoma - is it still appropriate to say that that these mice "never develop melanoma" as claimed?

We completely agree and apologize for this overstatement. Although the KP plot goes only until 6 months we followed the mice up to one year before terminating the experiment. During this period none of the mice developed melanoma. We therefore changed the text of page 6 from:

In contrast, *Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$* mice **never** developed melanoma, but instead a hair graying phenotype with normal skin morphology (Fig. 1).

to

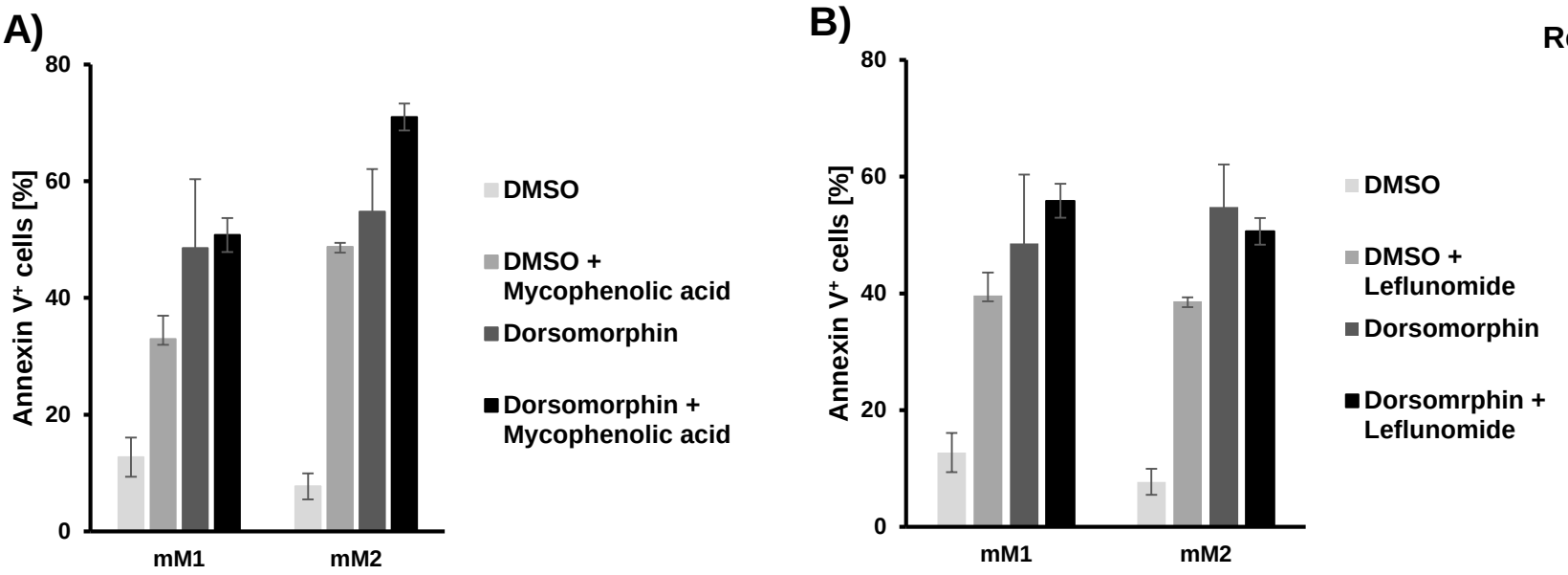
In contrast, *Tyr::Nras^{Q61K}INK4a$^{-/-}$c-Myc$^{\Delta/\Delta}$* mice did not developed **melanoma within the investigated time frame,** but instead a hair graying phenotype with normal skin morphology (Fig. 1).

3) In the section dealing with the levels of MYC-GFP expression in primary and metastatic sites, it would be better to refer to MycHigh and MycLow, rather than Myc+ and Myc-, for clarity.

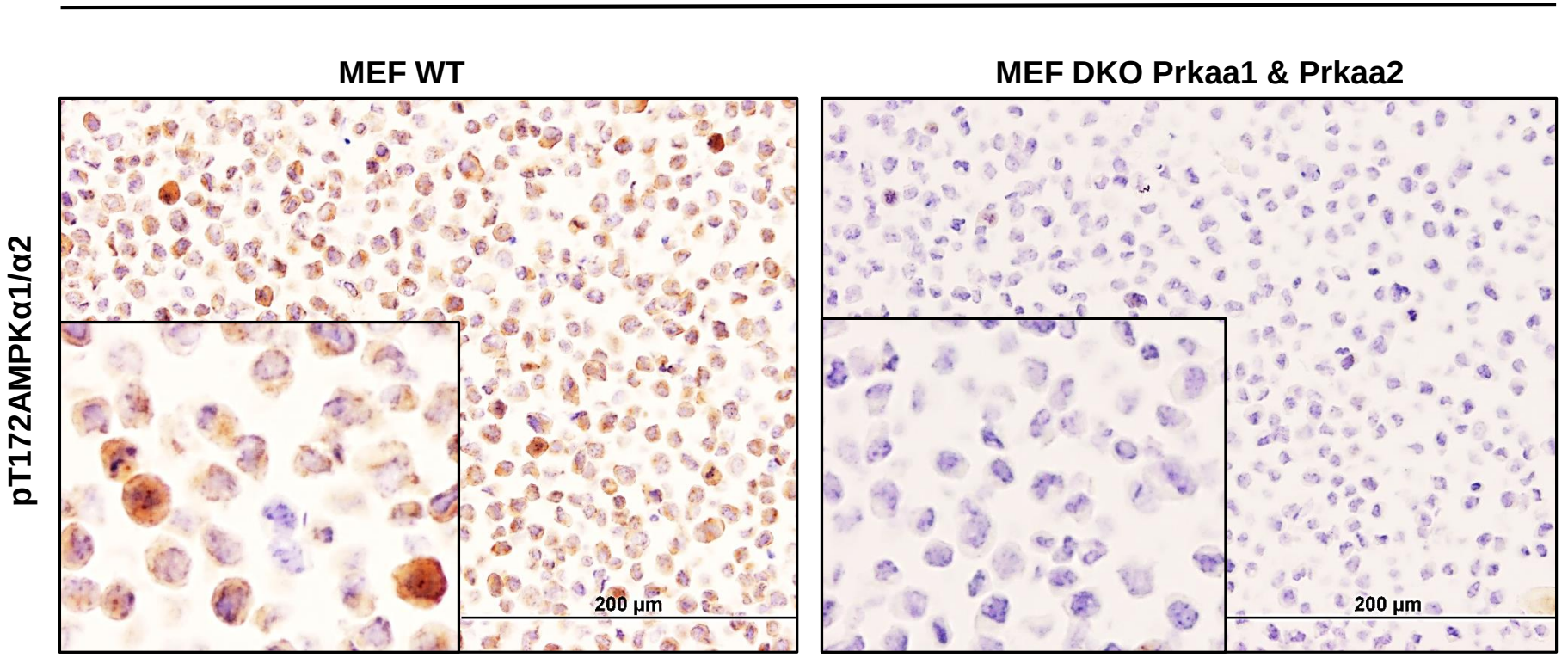
We agree and changed the text as well as Fig 5 accordingly.

4) The authors may wish to note that depletion of AMPKα1 was previously shown to be synthetic lethal with MYC overexpression in the study by Liu, Ulbrich et al (which they do cite).

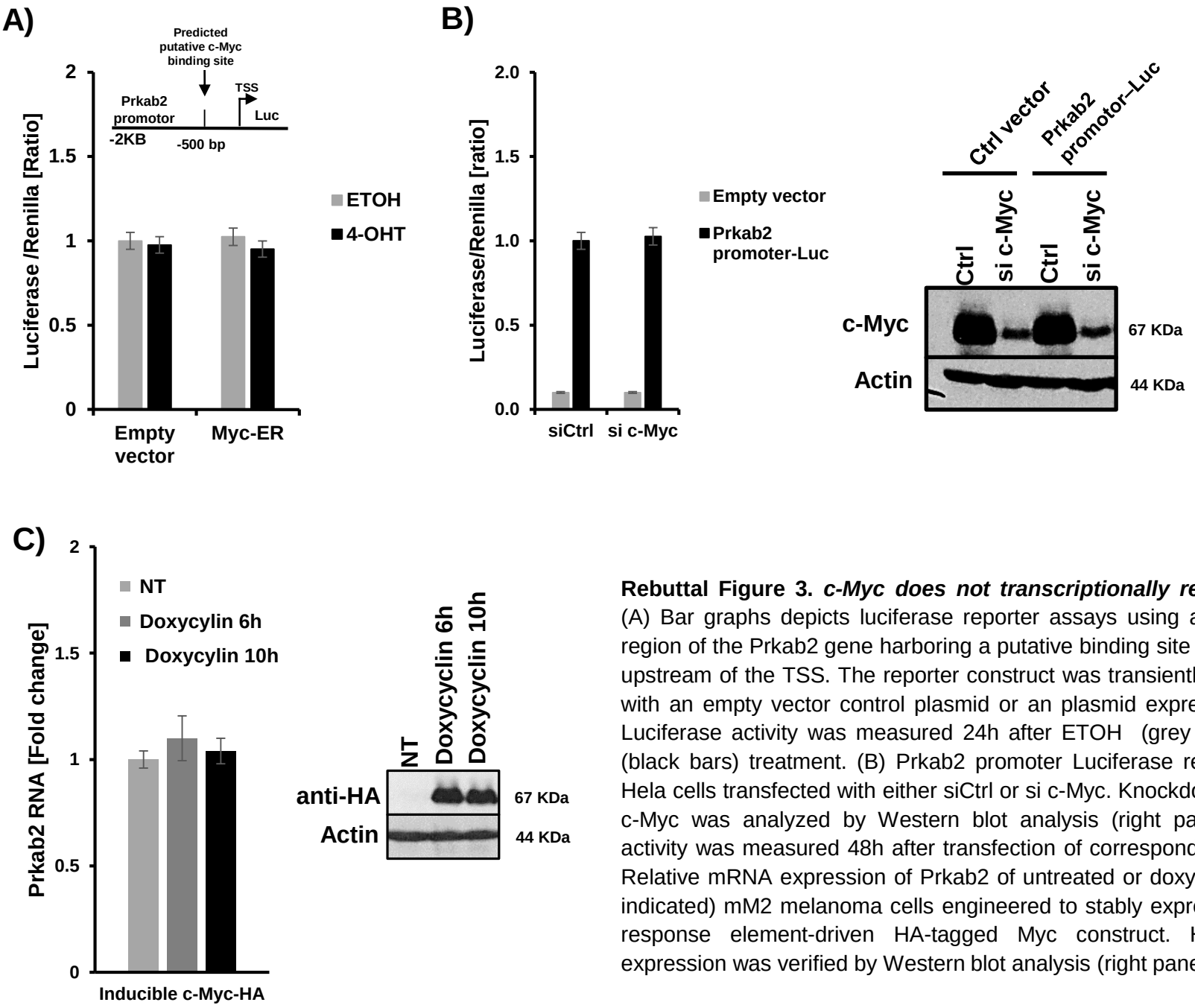
We thank the referee for pointing this out. We did cite the paper in our first version of the manuscript and now also investigated the status of ARK5 directly, which is not expressed in our melanoma cells. Please see point 2 above.



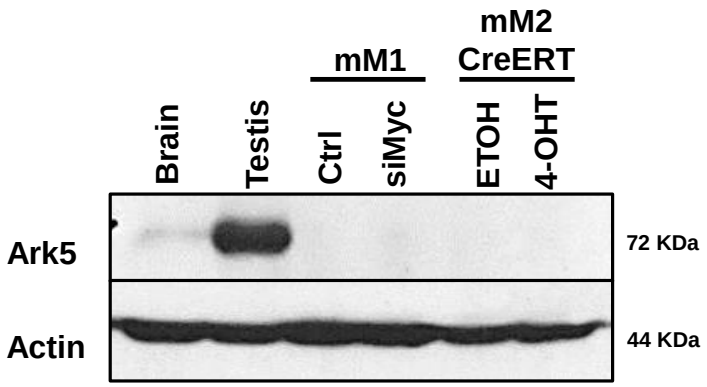
Rebuttal Figure 1. ***Nucleic acid synthesis inhibitors readily induce apoptosis in mM1 and mM2 cells.*** (A) Bar graph depicts quantification of apoptotic cells using AnnexinV/7AAD staining of mM1 and mM2 melanoma cells 48h post treatment – DMSO (light grey bar), DMSO+Mycophelonic acid (grey bar, 20µM), Dorsomorphin (dark grey bar, 10µM) and Mycophelonic acid+Dorsomorphin (black bar, 20µM+10µM, respectively). (B) Bar graph depicts quantification of apoptotic cells using AnnexinV/7AAD staining of mM1 and mM2 melanoma cells 48h post treatment – DMSO (light grey bar), DMSO + Leflunomide (grey bar, 20µM), Dorsomorphin (dark grey bar, 10µM) and Leflunomide + Dorsomorphin (black bar, 25µM+10µM, respectively). Concentrations of the respective inhibitors were titrated to observe an inhibitory effect on proliferation of melanoma cells with the least possible amount of apoptosis. Lower doses did not affect proliferation of melanoma cells. Data are presented as mean +/- s.d. of one representative out of two independent experiments. In each experiment, all samples were done in triplicates.



Rebuttal Figure 2. ***Specific pAMPK staining on MEFs.*** pAMPK immunostaining on MEFs either wilde type (WT; left panel) or double knockout (DKO) for *Prkaa1* and *Prkaa2* (right panel) treated with the AMPK activator AICAR as indicated. Insets show 40x magnification. Scale bars represent 200 µm.

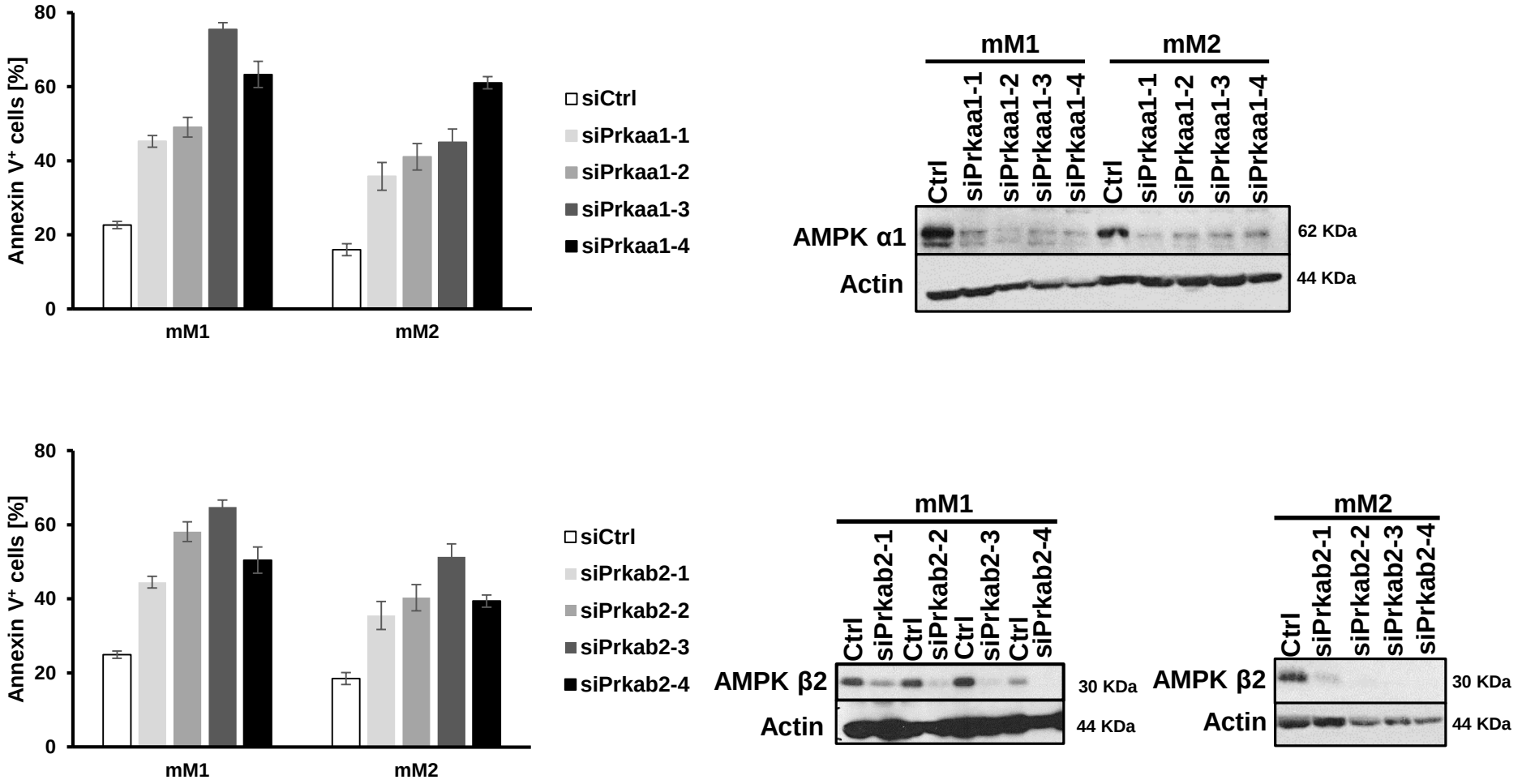


Rebuttal Figure 3. ***c-Myc does not transcriptionally regulate Prkab2.*** (A) Bar graphs depicts luciferase reporter assays using a 2 kb promoter region of the Prkab2 gene harboring a putative binding site for c-Myc 500bp upstream of the TSS. The reporter construct was transiently co-transfected with an empty vector control plasmid or an plasmid expressing Myc- ER. Luciferase activity was measured 24h after ETOH (grey bars) or 4-OHT (black bars) treatment. (B) Prkab2 promoter Luciferase reporter assay in Hela cells transfected with either siCtrl or si c-Myc. Knockdown efficiency of c-Myc was analyzed by Western blot analysis (right panel). Luciferase activity was measured 48h after transfection of corresponding siRNAs. (C) Relative mRNA expression of Prkab2 of untreated or doxycylin treated (as indicated) mM2 melanoma cells engineered to stably express a tetracyclin response element-driven HA-tagged Myc construct. HA-tagged Myc expression was verified by Western blot analysis (right panel).

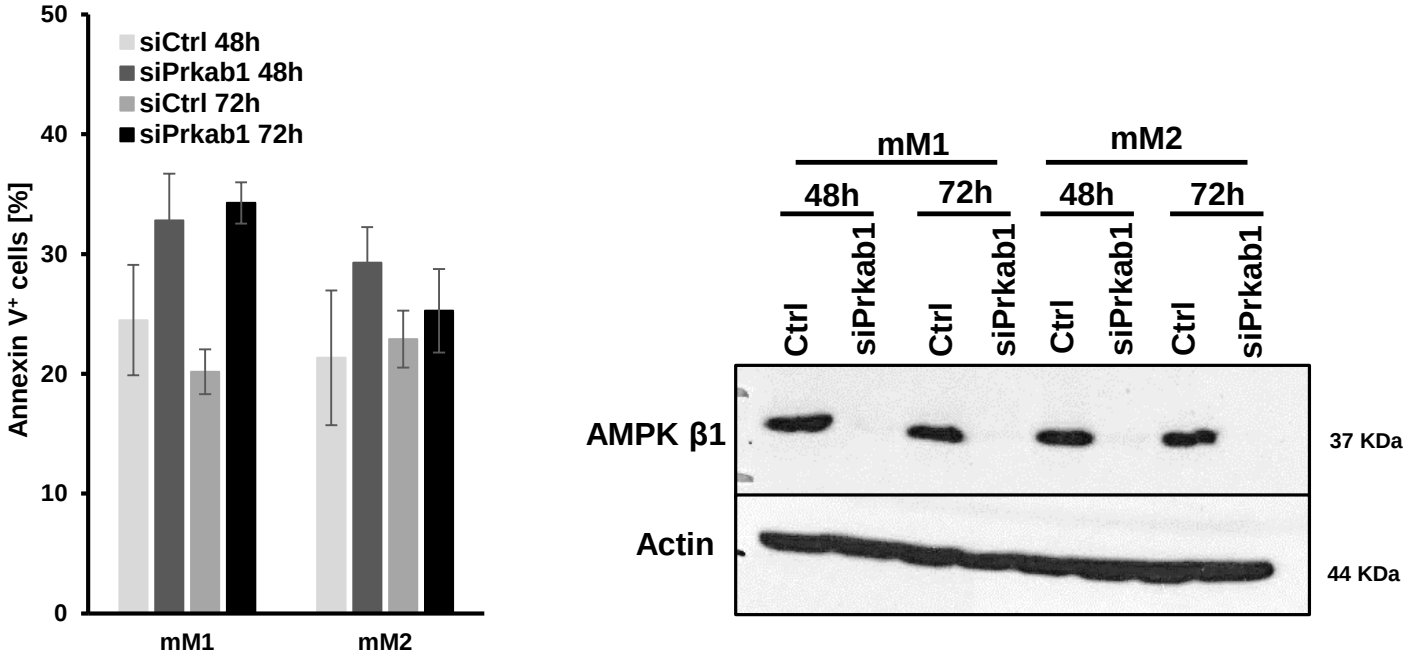


Rebuttal Figure 4. ***Primary murine melanoma cell lines do not express Ark5.*** Western blot analysis for Ark5 using protein extracts from brain and testis (as positive control) or mM1 and mM2 cells 48h after knockdown or genetic c-Myc depletion as indicated.

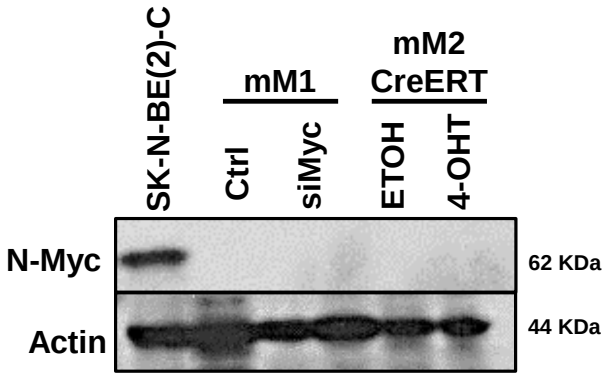
A)



Rebuttal Figure 5. ***Pool of single siRNAs targeting Prkaa1 induce apoptisis and downregulate AMPKα1.*** Bar graph depicts quantification of apoptotic cells using AnnexinV/7AAD staining of mM1 and mM2 melanoma cells 48h after siRNA mediated knockdown of Prkaa1 (A) and Prkab2 (B) using four different siRNA as indicated. Knockdown efficacy of individual siRNA was assessed by Western blot (right panels).



Rebuttal Figure 6. ***Knockdown of AMPKβ1 in mM1 and mM2 barely induces apoptosis.*** Bar graph depicts quantification of apoptotic cells using AnnexinV/7AAD staining of mM1 and mM2 melanoma cells 48h and 72h after siRNA mediated knockdown of Prkab1. The siRNA (smart pool) mediated knockdown efficacy was verified by Western blot analysis (right panel).



Rebuttal Figure 7. ***No redundancy between c-Myc and N-Myc in mM1 and mM2 melanoma cells.*** Western blot analysis for N-Myc using protein extracts from the neuroblastoma cell line SK-N-BE(2)-C (positive control) or mM1 and mM2 cells 48h after knockdown or genetic c-Myc depletion as indicated.

2nd Editorial Decision 08 January 2018

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has been seen once more by referees #2 and #3 (see comments below), and I am happy to inform you that they now support publication.

Before sending you an official acceptance letter, a few editorial points need to be taken care of.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we have received the revised version, we should be able to swiftly proceed with formal acceptance and production of the manuscript! Please let me know in case you have any questions.

REFEREE REPORTS

Referee #2:

All the points raised during my previous review have been addressed by the authors and the manuscript has much improved.

Referee #3:

The authors have addressed all of my concerns and the additional data significantly add to the manuscript. I believe that the manuscript is now suitable for publication in EMBO Journal

2nd Revision - authors' response 12 January 2018

Thank you for your decision letter of January 8th concerning our manuscript (EMBOJ- 2017-97673) entitled “AMPK promotes survival of c-Myc positive melanoma cells by suppressing oxidative stress” by Kfoury et al..
We thank all referees and editors for their constructive criticism, which allowed us to improve our data submission and hope that the revised version of our manuscript is now acceptable for publication in The EMBO Journal

3rd Editorial Decision 17 January 2018

Many thanks for sending the final version of your manuscript. I appreciate the introduced changes, and I am happy to accept your manuscript for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Freddy Radtke
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-97673

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 $\chi 2$ 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.

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all in vitro experiments two independent cell lines were used, two independent experiments were performed per cell line and each experimental sample was done in triplicates. For all in vivo animal studies, animal numbers were calculated in accordance with the 3R guidelines (http://www.3rs-reduction.co.uk/html/6__power_and_sample_size.html) using power analysis based on a Null hypothesis
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animal cohort size were calculated based upon group size as a function of signal to noise ratio with at least 0.03 significance, the alternative hypothesis being two-sided and the power calculation being 80% power.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In the animal studies no animals were excluded from the analysis.
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.	(a) Study personell was not blinded to the group allocation and assessing the outcome of animal studies. However, all mice and data collected were processed using the same procedures independently of their treatment received. (b) Rag2gc animals were age matched for the independent experiment (12-16wks of age) and equally distributed for sex between the different treatment groups.
For animal studies, include a statement about randomization even if no randomization was used.	Rag2gc were randomly pooled according to sex from an established breeding colony. Otherwise, no randomization was performed.
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.	(a) RAG2gc inbred animals were randomly chosen from an established breeding colony - see also 4.b (b) Research personell performing the immunostaining on human melanoma biopsies were blinded for sample staining and interpretation
4.b. For animal studies, include a statement about blinding even if no blinding was done	Research personell was not blinded in the animal studies. Nevertheless, all mice and data collected were processed using the same procedures independently of their treatment received.
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical data meets the assumptions. Statistical significance was assessed using Students t-test as indicated in Figure legends; in Kaplan-Meyer plots p-values were assessed using LOG-RANK statistical test.
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tum

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody list and nucleotide sequences are provided in supplementary information.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	In house derived mouse melanoma cell lines: mM1 and mM3 were derived from melanoma bearing Tyr::NrasQ61KINK4a-/- mice. mM2 was derived from a melanoma bearing Tyr::NrasQ61KINK4a-/-c-Myc lox/lox mice. B16-F10 murine melanoma cells were originally purchased from ATCC. All human melanoma cell line were obtained from the Institute of Pathology, University Hospital of Lausanne or the Ludwig Institute for Cancer Resarch, Lausanne Branch. Cell lines used in this study have been tested for mycoplasma contamination and confirmed negative.

* 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.	Tyr::NrasQ61K INK4a-/- mice were previously described (Ackermann et al, 2005) and crossed to c-Myclox/lox (Trumpp et al, 2001) and c-MycG/G (Huang et al, 2008) to generate Tyr::NrasQ61KINK4a-/-c-Myclox/lox and Tyr::NrasQ61KINK4a-/-c-MycG/G, respectively. Tyr::NrasQ61KINK4a-/-c-Myclox/lox were crossed to a melanocyte specific Cre line – Tyr::Cre (Delmas et al, 2003) to generate Tyr::NrasQ61K INK4a-/-c-MycΔ/Δ. Rag2yc-/- (B6.Rag2tm1FwaII2gtmWjl) were purchased from the TACONIC (United States).All animal work was conducted according to Swiss national guidelines. All mice were kept in the animal facility under EPFL animal care regulations. This study has been reviewed and approved by the cantonal veterinary office - VD3273.1 - Additionally refer to text
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This study has been reviewed and approved by the cantonal veterinary office."Servie de la consommation et des Affaires vétérinaires (SCAV). Authorization number: VD3273
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.	Animal experimentations were done in compliance with ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Our RNA seq.data have been uploaded to GEO NCBI. Gene Expression Omnibus GSE108447
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 as our RNA seq.data have been uploaded to GEO NCBI. Gene Expression Omnibus GSE108447
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A