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Tuberous sclerosis complex is required for tumor maintenance in MYC-driven Burkitt's lymphoma

Götz Hartleben, Christine Müller, Andreas Krämer, Heiko Schimmel, Laura M. Zidek, Carsten Dornblut, René Winkler, Sabrina Eichwald, Gertrud Kortman, Christian Kosan, Joost Kluiver, Iver Petersen, Anke van den Berg, Zhao-Qi Wang and Cornelis F. Calkhoven

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Transaction Report:

(Note: An earlier version of this manuscript was assessed by another journal and was then transferred to The EMBO Journal. As the original review of the manuscript was performed outside of The EMBO Journal's transparent review process policy, this Peer Review information is not included here. 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.)

Editorial Correspondence 15th Dec 2017

Thank you again for submitting you manuscript EMBOJ-2017-98589, "TSC1 is required for tumor maintenance in MYC-driven Burkitt's lymphoma", to our journal. We have now received comments from two arbitrating referees, which I am enclosing below for your information. While the reports express some interest in the work, I am afraid that despite the previous responses and revisions available to them, neither referee considered the paper ready for EMBO Journal publication in the present form. Even though there may be some confusion in referee 1's reading of the Myc-miR-15-TSC1 axis, both referees bring up several apparently well-taken points to address prior to publication.

I realize that after the significant revision efforts you already spent on this study, you may not be prepared to embark on further follow-up investigations here, and that you also may have already attempted some of the requested experiments in the past. Therefore, I would at this point like to give you the opportunity to consider the referees' comments and provide a tentative response letter detailing which further experiments you would be willing/able to undertake in order to address the referees' concerns, or how else you could envision answering their comments. Based on such a draft response, we could then work out whether or not it would be reasonable to invite and pursue a revision of this study for The EMBO Journal (or, possibly, one of our sister journals). I would therefore appreciate if you could confer with your coworkers and send us such a response at your earliest convenience, ideally over the course of next week. Should you have any further questions in this regard, of course please do not hesitate to let me know.

REFEREE COMMENTS:

Arbitrating Referee #1

The manuscript by Hartleben et al. reports the intriguing observation that MYC (Bukitt lymphoma and a MYC-inducible B cell P493 lymphoma model) represses TSC1 via miR-15a to attenuate mTOR activity. The evidence suggests fine-tuning of mTOR activity, such that excessive mTOR activity is incompatible with high MYC levels in several model systems. The authors have satisfied most previous reviewers with additional experimental evidence. This story has been presented orally publically previously, and it is commendable that the authors persisted to publish their intriguing findings that counters some prevailing concepts. It appears that the authors have cautiously nuanced their narrative regarding the interplay between MYC and mTOR. There are several items that would improve the scholarship of this manuscript.

1. The authors omit referring to the work of E. Schmidt showing that MYC could repress TSC2 in certain context: Ravitz et al. Cancer Res 2007. For completeness, this foundational work should be discussed.
2. Amcheslavsky et al. (JCB, 193:695 (2011)) suggests a similar interaction between MYC and TSC2 in the growth and proliferation of midgut cells in adult Drosophila. In particular, these authors show that excessive TOR-mediated growth (loss of TSC2) inhibits cell division unless dMYC levels were lowered.
3. Reviewer 4 has an important point:
"In the original question #2, the reviewer pointed out that, in U2OS MycER cells, the authors showed that TSC1 knockdown led to synthetic lethality under Myc induction condition, however, in this same cell line, Myc induction did not even affect TSC1 expression.

In the rebuttal letter, the authors argue that they have provided plenty of other evidences that Myc can induce TSC1 expression (as shown in Fig 1), and they simply use U2OS cells (in which Myc does not affect TSC1 expression) to demonstrate the synthetic lethality (Note that even the validity of this conclusion is challenged in question 1).

In this reviewer's opinion, there is a logical issue here which undermines their hypothesis that induction of TSC1 by deregulated c-Myc is required for survival of c-Myc expressing cancer cells (as stated in the last paragraph of page 6): if Myc does not induce TSC1 expression, how would they explain the dependency of Myc high cells on TSC1 for survival? Again, the reviewer argue that this actually suggest that TSC1 is important for survival in both Myc-low and Myc-high cells (not specific for Myc high cells)."

I believe that these are important issues, particular with the use of the U2OS MYC-ER system. The authors did not perform any time course experiments to document how MYC in this system affects mTOR signaling through immunoblots of mTOR, p-TOR, S6, p-S6, 4EBP, and p-4EBP. Hence, these experiments should be discussed with caution, particularly, since U2OS cells do not tolerate MYC over-expression beyond 4-5 days. Whether nutrient deprivation from uncontrolled MYC and increased mTOR activity contribute to death is not addressed.

Arbitrating Referee #2

Hartleben et. al. show a requirement for TSC1 in MYC-driven lymphoma. They use cell lines, xenograft models and patient data to show that MYC-high lymphomas have high TSC1 levels, needed for maintenance of the tumors. The authors show that MYC upregulates TSC1 by increasing TSC1 mRNA stability. Moreover, knock down of TSC1 in cells with high levels of MYC causes apoptosis by increasing mitochondrial respiration and ROS generation. Specific comments on the manuscript are as follows:

1. The authors show that MYC acts primarily through miR-15 to affect TSC1 levels. They should determine the level of miRNA-15 in their panel of low MYC cell lines. In support of their claim of an MYC-miR15-TSC1 axis, miR-15 expression should inversely correlate with MYC expression. The authors should also test for cell viability upon induction of miR-15 in a high MYC background.
2. To determine whether the effect of TSC1 KD in high MYC cell lines is dependent on the TSC1-TSC2 complex-mTOR signaling axis, i.e., not an mTOR independent effect, the authors should

knock down TSC2 and obtain results similar to the TSC1 KD. These experiments would further solidify their mechanism that TSC1 actions are via the TSC1-TSC2 complex.
3. Upon knockdown of TSC1 in high MYC expressing cells, the authors see upregulation of mTORC1 signaling (as expected). Such upregulation could inhibit AKT through the negative feedback loop in the mTORC1 pathway. As AKT is known to promote cell survival, its reduction in high MYC cells could cause cell death. The authors should explore this possibility by examining AKT levels in the cells in which they KD TSC1 and include the results in the manuscript. An effect through AKT could dramatically change their model.

Authors' Response - Additional Correspondence 20th December 2017

Thank you very much for considering our manuscript. I am really grateful for the time you and the reviewers spend on this. We have dealt with the reviewer's comments in detail. Please see the attached word file. For some comments we already can provide data, for others the reviewer simply overlooked data that are already in. Those issues that are left can be easily addressed experimentally, although – will all respect - we doubt that they will improve the paper or change the conclusion (we try to explain in the replies). That is why I phrase like "if the reviewer thinks it is required we do the experiment".

Arbitrating Referee #1

The manuscript by Hartleben et al. reports the intriguing observation that MYC (Bukitt lymphoma and a MYC-inducible B cell P493 lymphoma model) represses TSC1 via miR-15a to attenuate mTOR activity. The evidence suggests fine-tuning of mTOR activity, such that excessive mTOR activity is incompatible with high MYC levels in several model systems. The authors have satisfied most previous reviewers with additional experimental evidence. This story has been presented orally publically previously, and it is commendable that the authors persisted to publish their intriguing findings that counters some prevailing concepts. It appears that the authors have cautiously nuanced their narrative regarding the interplay between MYC and mTOR. There are several items that would improve the scholarship of this manuscript.

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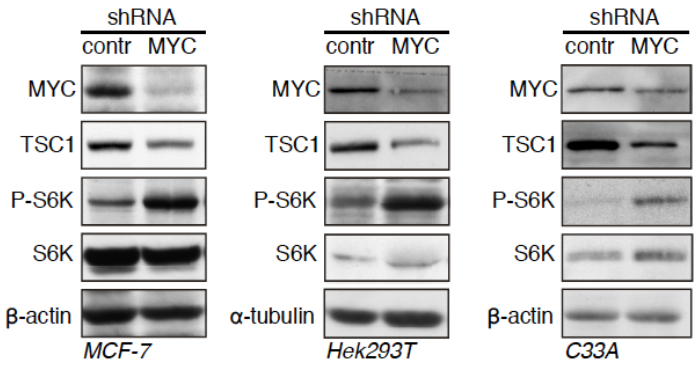
We will discuss this paper in a revised version of the manuscript.

We do show high expression of TSC2 together with TSC1 in high MYC expressing BL cells (cell lines: Fig. 1A, B, C, D, E, patient tumors: 2B).

To strengthen the MYC-TSC1/2 regulation beyond Burkitt's lymphoma we have data on MYC knockdown and TSC1-S6K(-P) analysis in MCF7 (breast cancer), HEK293T (embryonic kidney) and C33A (cervix carcinoma) cell lines showing that reduction of MYC results in decreased TSC1 expression and enhanced mTORC1 signaling (increase in S6K-P). **We can include these data.**



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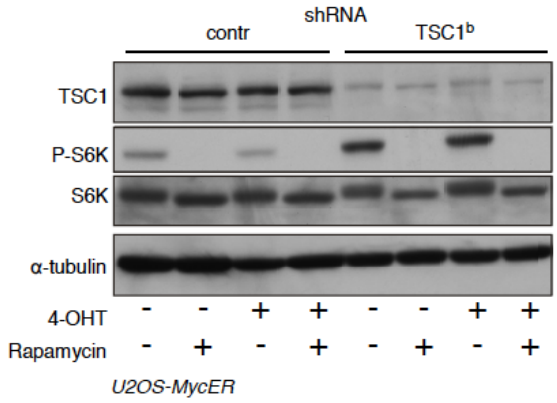
We will discuss this paper in a revised version of the manuscript.

Although the biological context (Drosophila) and consequence (inhibition of cell division) of TOR hyperactivation is different, also here high MYC levels are incompatible with high TOR activation, supporting that this is a more general phenomena.

3. Reviewer 4 has an important point:

"In the original question #2, the reviewer pointed out that, in U2OS MycER cells, the authors showed that TSC1 knockdown led to synthetic lethality under Myc induction condition, however, in this same cell line, Myc induction did not even affect TSC1 expression.

We have blots showing expression of TSC1, S6K/S6K-P and tubulin loading control. Notably, U2OS is not a BL cell line. We do not claim that TSC1 expression is under the control of MYC in all cell types, but that also in such an occasion cell survival depends on the presence of TSC1 under high MYC expression, and that cell survival can be rescued under TSC1 KD condition either by lowering MYC or inhibition of mTORC1 by rapamycin treatment (shown in Figure 3C).



In the rebuttal letter, the authors argue that they have provided plenty of other evidences that Myc can induce TSC1 expression (as shown in Fig 1), and they simply use U2OS cells (in which Myc does not affect TSC1 expression) to demonstrate the synthetic lethality (Note that even the validity of this conclusion is challenged in question 1).

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As a reaction on this comment we added data showing that TCS1 KD does not affect survival in MYC low HL cells (Fig. EV3E).

We do explain, why high MYC cells depend on TSC1 (even if the expression is not always under its control): **it is needed to suppress mTORC1 to lower ROS production (Fig. E-G)(!)**

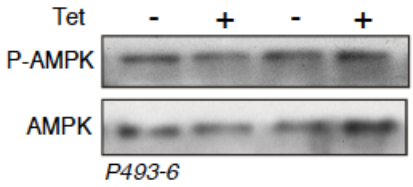
I believe that these are important issues, particular with the use of the U2OS MYC-ER system. The authors did not perform any time course experiments to document how MYC in this system affects mTOR signaling through immunoblots of mTOR, p-TOR, S6, p-S6, 4EBP, and p-4EBP.

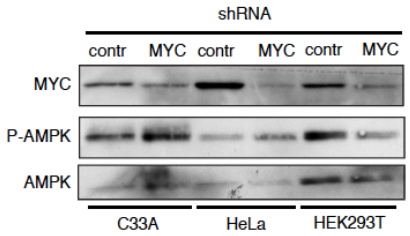
Hence, these experiments should be discussed with caution, particularly, since U2OS cells do not tolerate MYC over-expression beyond 4-5 days. Whether nutrient deprivation from uncontrolled MYC and increased mTOR activity contribute to death is not addressed.

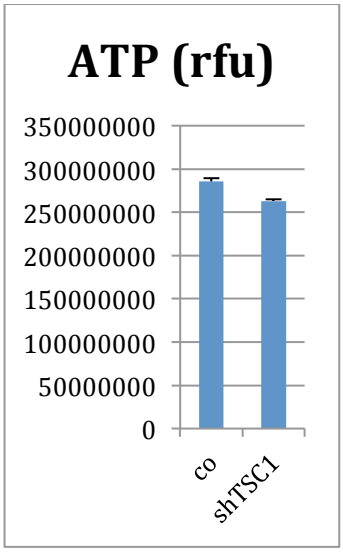
Maybe the best solution is to improve the description of this experiment and more cautiously phrase the conclusion?

Alternatively, we could remove these data because of the known incompatibility of U2OS with high-MYC expression for longer time, which makes interpretation rather difficult.

The Murphy lab (Liu et al 2012 Nature 483, 608) showed in U2OS cells that uncontrolled MYC and mTORC1 activation leads to energetic stress, however, we did not observe this in Burkitt´s lymphoma cell lines: in p493-6 cells we did not observe a change in AMPK-phosphorylation upon induction of MYC (-Tet). In three other cell high MYC levels only correlated with increased AMPK-phosphorylation in HEK293T cells. Also the ATP levels in P493-6 cells did not dramatically change in TSC1 KD cells (8% less).







A time course experiment has been published by Eilers/Murphy labs (please see figure 2d from Liu et al). **If the reviewer thinks a time course experiment is still required in addition we are happy to perform this experiment.**

Arbitrating Referee #2

Hartleben et. al. show a requirement for TSC1 in MYC-driven lymphoma. They use cell lines, xenograft models and patient data to show that MYC-high lymphomas have high TSC1 levels, needed for maintenance of the tumors. The authors show that MYC upregulates TSC1 by increasing TSC1 mRNA stability. Moreover, knock down of TSC1 in cells with high levels of MYC causes apoptosis by increasing mitochondrial respiration and ROS generation. Specific comments on the manuscript are as follows:

1. The authors show that MYC acts primarily through miR-15 to affect TSC1 levels. They should determine the level of miRNA-15 in their panel of low MYC cell lines. In support of their claim of an MYC-miR15-TSC1 axis, miR-15 expression should inversely correlate with MYC expression. The authors should also test for cell viability upon induction of miR-15 in a high MYC background.

The reviewer may have missed out the most important data on this presented in Table EV1 that was retrieved from a paper from our co-authors (Robertus et al 2010 BJH 149, 896; see attached PDF). miR-15a is also shown by the Mendell lab (Chang et al (2008) Nat Genet 40, 43) to be suppressed by MYC.
If the reviewer feels the extra analysis is required we can analyze miR-15a levels in KMH2 and L540 low MYC cell lines.

We can examine cell viability after miR-15 overexpression.
We do show that miR-15a overexpression increases oxygen consumption (OCR) in Fig. 5H.
The question is if miR-15a will be sufficient. As we show and discuss (and others showed) other miRs are suppressed by MYC that have seed-sequences in the TSC1-3'UTR (Table EV1 AND Fig. EV5A).

2. To determine whether the effect of TSC1 KD in high MYC cell lines is dependent on the TSC1-TSC2 complex-mTOR signaling axis, i.e., not an mTOR independent effect, the authors should knock down TSC2 and obtain results similar to the TSC1 KD. These experiments would further solidify their mechanism that TSC1 actions are via the TSC1-TSC2 complex.

We can perform the proposed TSC2 KD experiments.

3. Upon knockdown of TSC1 in high MYC expressing cells, the authors see upregulation of mTORC1 signaling (as expected). Such upregulation could inhibit AKT through the negative feedback loop in the mTORC1 pathway. As AKT is known to promote cell survival, its reduction in high MYC cells could cause cell death. The authors should explore this possibility by examining AKT levels in the cells in which they KD TSC1 and include the results in the manuscript. An effect through AKT could dramatically change their model.

The reviewer means the mTORC1-S6K dependent IRS1-phosphorylation (negative insulin signaling feedback) resulting in decrease in AKT-Thr308-phosphorylation. However, in B cells mTORC1 is thought to be activated through B-cell receptor signaling, and it seems not to be known if IRS1 plays a role as in insulin sensitive cells.
Nevertheless, we can perform AKT-Thr308-phosphorylation analysis in TSC1 KD cells.

Also in case of AKT contributing to the reduced cell survival our results show that the increased respiration and ROS production under high MYC and mTORC1 signaling can be reverted by either rapamycin treatment or MYC suppression and restores cell survival (Fig. 4E-G). Thus, our model will not change “dramatically”.

1st Editorial Decision 21st Dec 2017

Thank you very much for your response letter and proposal for revising your manuscript in response to the arbitrating referees' comments. I am happy to read that you seem to be in a position to answer the remaining points with new data/experiments and/or additional clarifications. Therefore, I would like to invite you to prepare a revised version as outlined in your response letter. Regarding the comments of arbitrator 1, please incorporate the data you already have as suggested, while for his/her last point, better description and discussion should indeed be sufficient and no data removal nor additional time course experiment required in light of your clarifications. On the other hand, I think incorporating that all proposed experiments answering to arbitrator 2's points would indeed be helpful to back up the study as it stands. With these revisions and additional textual clarifications, we should be ready to consider the paper further for eventual publication in The EMBO Journal.

Revision Response to Arbitrating Referees 29th June 2018

Dear Reviewers, dear editor,

Thank you for considering our revised manuscript and for you patience. We have addressed all your concerns and you will find a point-to-point below. All changes and new figure annotations in the text are in red.

We hope you now will find the accumulated evidence strong enough to be published in EMBO Journal.

We propose to make a small change in the title:

Tuberous sclerosis complex is required for tumor maintenance in MYC-driven Burkitt's lymphoma

(In stead of: TSC1 is required for tumor maintenance in MYC-driven Burkitt's lymphoma)

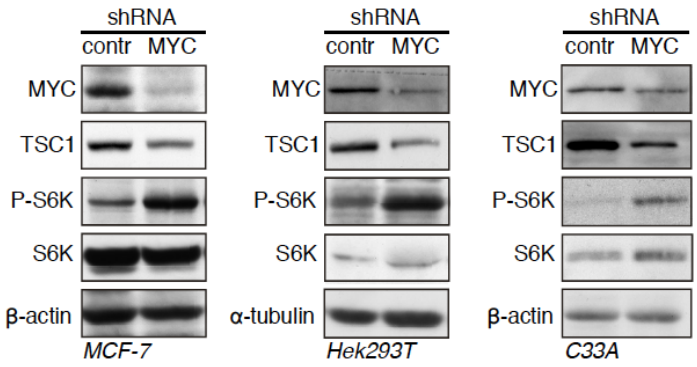
Arbitrating Referee #1

The manuscript by Hartleben et al. reports the intriguing observation that MYC (Bukitt lymphoma and a MYC-inducible B cell P493 lymphoma model) represses TSC1 via miR-15a to attenuate mTOR activity. The evidence suggests fine-tuning of mTOR activity, such that excessive mTOR activity is incompatible with high MYC levels in several model systems. The authors have satisfied most previous reviewers with additional experimental evidence. This story has been presented orally publically previously, and it is commendable that the authors persisted to publish their intriguing findings that counters some prevailing concepts. It appears that the authors have cautiously nuanced their narrative regarding the interplay between MYC and mTOR. There are several items that would improve the scholarship of this manuscript.

1. The authors omit referring to the work of E. Schmidt showing that MYC could repress TSC2 in certain context: Ravitz et al. Cancer Res 2007. For completeness, this foundational work should be discussed.
We now discuss this paper in at page 11 that shows TSC2 transcriptional downregulation by Myc.in Rat1A-based rat fibroblasts.

We do observe high expression of TSC2 together with TSC1 in high MYC expressing BL cells compared to low TSC1 and TSC2 expression in low MYC HL cells (cell lines: Fig. 1A, B, C, E, patient tumors: 2B).

To strengthen the MYC-TSC regulation beyond Burkitt's lymphoma we have data on MYC knockdown and TSC1-S6K(-P) analysis in MCF7 (breast cancer), HEK293T (embryonic kidney) and C33A (cervix carcinoma) cell lines showing that reduction of MYC results in decreased TSC1 expression and enhanced mTORC1 signaling (increase in S6K-P).
Since they are not related to Burkitt's lymphoma, we choose not to include the data in the manuscript but to show these data here. If the reviewer wishes we will of course include them in the manuscript.



2. Amcheslavsky et al. (JCB, 193:695 (2011)) suggests a similar interaction between MYC and TSC2 in the growth and proliferation of midgut cells in adult Drosophila. In particular, these authors show that excessive TOR-mediated growth (loss of TSC2) inhibits cell division unless dMYC levels were lowered.

We now discuss this paper at page 11.
Although the biological context (Drosophila) and consequence (inhibition of cell division) of TOR hyperactivation is different, also here high MYC levels are incompatible with high TOR activation, supporting that this is a more general phenomena. We now also show that knockdown of TSC2 similar to TSC1 does raise mitochondrial respiration and ROS production and decreases cell survival in BL cell lines (Figures EV3G EV4F)

3. Reviewer 4 has an important point:
"In the original question #2, the reviewer pointed out that, in U2OS MycER cells, the authors showed that TSC1 knockdown led to synthetic lethality under Myc induction condition, however, in this same cell line, Myc induction did not even affect TSC1 expression.
In the rebuttal letter, the authors argue that they have provided plenty of other evidences that Myc can induce TSC1 expression (as shown in Fig 1), and they simply use U2OS cells (in which Myc does not affect TSC1 expression) to demonstrate the synthetic lethality (Note that even the validity of this conclusion is challenged in question 1).

The U2OS cell line is not a BL cell line. We do not claim that TSC1 expression is under the control of MYC in all cell types, but that also in such an occasion cell survival depends on the presence of TSC1 under high MYC expression, and that cell survival can be rescued under TSC1 KD condition either by lowering MYC or inhibition of mTORC1 by rapamycin treatment (as shown in Fig. 3C).

In this reviewer's opinion, there is a logical issue here which undermines their hypothesis that induction of TSC1 by deregulated c-Myc is required for survival of c-Myc expressing

cancer cells (as stated in the last paragraph of page 6): if Myc does not induce TSC1 expression, how would they explain the dependency of Myc high cells on TSC1 for survival? Again, the reviewer argue that this actually suggest that TSC1 is important for survival in both Myc-low and Myc-high cells (not specific for Myc high cells)."
We have specified in the parapgraph, page 8 “BL cancer cells”

As a reaction on this concern we added data showing that TCS1 KD does not affect survival in MYC low HL cells (Fig. EV3F).
We do explain, why high MYC cells depend on TSC1 (even if the expression is not always under its control): it is needed to suppress mTORC1 to lower ROS production (Fig. 4E-G).

The Murphy/Eilers lab (Liu et al 2012 Nature 483, 608) showed that in U2OS cells high Myc expression and inhibition of the NUAK1/ARK5-AMPK pathway is synthetic lethal through activation of mTORC1. AMPK inhibits mTORC1 through activating the TSC complex by phosphorylation of TSC2 (Inoki et al 2003 Cell 115, 577). Thus, it is not surprising that TSC1 knockdown in U2OS cells with high MYC activity reduces cell viability similarly to the inhibition of the NUAK/ ARK5-AMPK pathway. However, as the Murphy/Eilers lab showed, in U2OS cells a high MYC activity downregulates mTORC1 activity via the induction of AMPK function und thus via a different mechanism as in BL cells.

I believe that these are important issues, particular with the use of the U2OS MYC-ER system. The authors did not perform any time course experiments to document how MYC in this system affects mTOR signaling through immunoblots of mTOR, p-TOR, S6, p-S6, 4EBP, and p-4EBP.

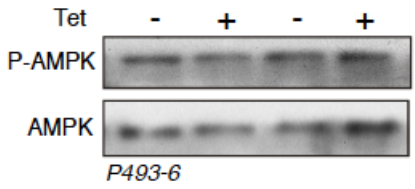
We now included analysis of P-S6K/S6K showing that mTORC1 signaling is decreased upon MYC induction. TSC1 KD, even in the presence of activated MYC, leads to increased mTORC1 activity in the U2OS MYC-ER system, in Fig. EV3A

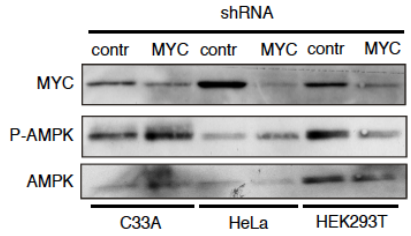
In addition, a time course experiment has been published by Eilers/Murphy labs (please see figure 2d from Liu et al).

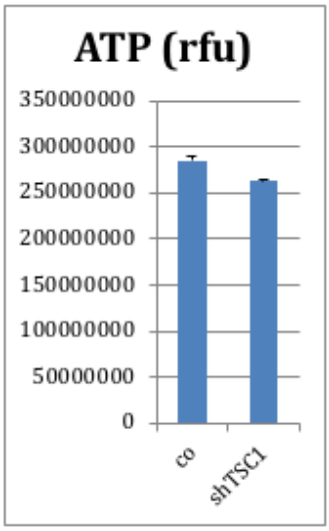
Hence, these experiments should be discussed with caution, particularly, since U2OS cells do not tolerate MYC over-expression beyond 4-5 days. Whether nutrient deprivation from uncontrolled MYC and increased mTOR activity contribute to death is not addressed.

The Murphy/Eilers lab (Liu et al 2012 Nature 483, 608) showed in U2OS cells that uncontrolled MYC and mTORC1 activation leads to energetic stress, however, we did not observe this in Burkitt´s lymphoma cell lines: in p493-6 cells we did not observe a change

in AMPK-phosphorylation upon induction of MYC (-Tet). When testing three other cell lines (C33A, HeLa and HEK293T) high MYC levels only correlated with increased AMPK-phosphorylation in HEK293T cells. Also the ATP levels in P493-6 cells did not dramatically change in TSC1 KD cells (8% less). We choose to show these data here. If the reviewer wishes we will of course include them in the paper.







Arbitrating Referee #2

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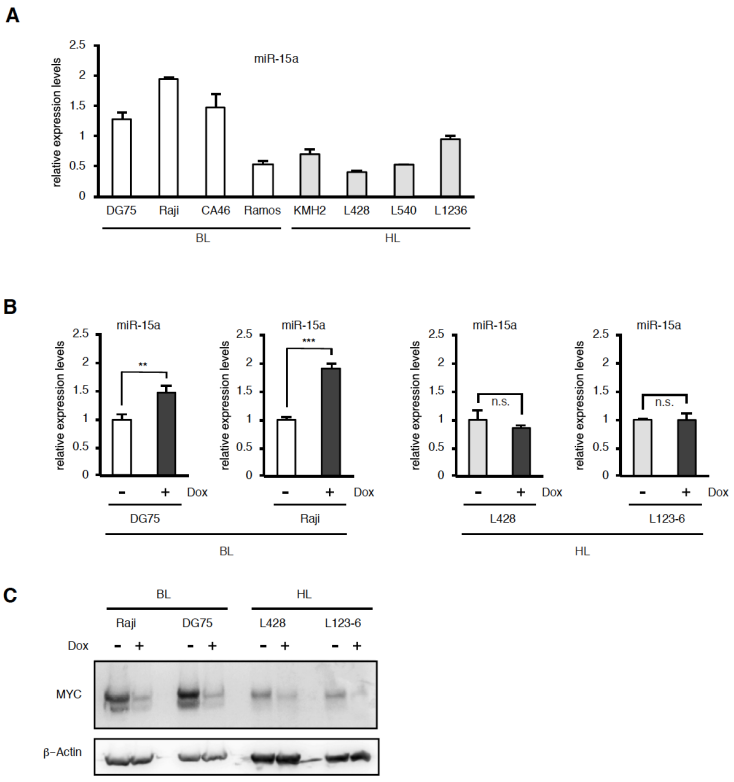
We describe a dual mechanism of TSC1 regulation. First, MYC transcriptionally controls TSC1 expression, and second, additionally represses several miRs that can downregulate TSC1 (Table EV1 and Fig. EV5A). miR-15a has the strongest effect compared to the other miRs, but there is likely an additive effect of all miRs.

We compared the expression levels of miR-15a between high MYC BL and low MYC HL cells, but we did not observed higher miR15a levels in the tested HL cells, despite the difference in TSC1 levels (see Fig. A below). Nevertheless, miR-15a is under control of MYC only in BL cells, and manipulation of MYC levels in HL cells has no effect on miR-15a levels (Fig. B and C below). Therefore, the MYC-miR15a-TSC1 axis seems to be exclusively important in high MYC BL cells. BL cells probably adopted this axis as a control mechanism due to the very high MYC levels in order to keep mTORC1 in check. In cells with lower MYC levels, like the here tested HL cells, this axis is of less importance with no need to balance MYC and mTORC1 activity, so MYC does not take control over miR15-TSC1. This is also reflected in the low TSC1 levels in these cells.
Because the miR expression data in HL versus BL cell lines are inconclusive we did not included them in the manuscript.



In addition, we now show that miR-15a overexpression results in reduced BL cell viability (Fig. EV5B). This is in line with the work of others, that miR-15a is a tumour suppressor in lymphomas (Cimmino et al (2005) PNAS 102, 13944).

Please also see the data on this presented in Table EV1 that was retrieved from a paper from our co-authors (Robertus et al 2010 BJH 149, 896: doi:10.1111/j.1365-2141.2010.08111.x). In addition, miR-15a was also shown by the Mendell lab to be suppressed by MYC (Chang et al (2008) Nat Genet 40, 43).

2. To determine whether the effect of TSC1 KD in high MYC cell lines is dependent on the TSC1-TSC2 complex-mTOR signaling axis, i.e., not an mTOR independent effect, the authors should knock down TSC2 and obtain results similar to the TSC1 KD. These experiments would further solidify their mechanism that TSC1 actions are via the TSC1-TSC2 complex.

We added the following data for TSC2 knockdown.
Fig. EV3F: TSC2 knockdown BL cells show a similar decrease in cell viability as upon TSC1 knockdown.
Fig. EV4F: TSC2 knockdown results in increased mitochondrial respiration and ROS production, similar to TSC1 knockdown.
In addition, our experiment showing that TSC1 KD induced cell death can be rescued by rapamycin (Fig. 3B) indicates that cell death is mediated through mTORC1.
Together, the results support our model where the TSC1/2 complex is required to control mTORC1 and secure survival in high MYC BL cells.

3. Upon knockdown of TSC1 in high MYC expressing cells, the authors see upregulation of mTORC1 signaling (as expected). Such upregulation could inhibit AKT through the negative feedback loop in the mTORC1 pathway. As AKT is known to promote cell survival, its reduction in high MYC cells could cause cell death. The authors should explore this possibility by examining AKT levels in the cells in which they KD TSC1 and include the results in the manuscript. An effect through AKT could dramatically change their model.

We performed the suggested experiments, shown in Fig. EV4A. TSC1 knockdown in BL cells leads to an increased phosphorylation of Ser-493 of AKT, reflecting higher activity. We tried hard to show Thr-308 phosphorylation as well but failed to do so both in TSC1 knockdown and control cells (although we can detect pan AKT).
Together with our rapamycin and antioxidant rescue experiments (Fig. 3B, E; 4A, C-G) altogether our data suggest that mTORC1 hyperactivation and not decreased AKT activity is responsible for the increased cell death. Possibly the increase in Ser-493 AKT phosphorylation is a secondary compensatory effect to counteract the cell death by mTORC1 hyperactivation.

2nd Editorial Decision 30th Aug 2018

Thank you for submitting your final revised manuscript. I am sorry for the delay in dealing with it, but I have now finally had a chance to carefully look through your responses and new data, and I am happy to let you know that I see no further objections towards publication. We have therefore now accepted it 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: Cornelis F. Calkhoven
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98589R

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	It was chosen baseon on earlier experiments performed in the lab using the same technique
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The animal experiment (xenograft) was performed through a company and the sample size estimate was based on their professional experience
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals or samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	if applicable, samples were not allocated by name but by numbering
For animal studies, include a statement about randomization even if no randomization was used.	The single animal experiment (xenograft) was performed by a company and was not randomized
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.	Part of the experiments were performed by technical staff members who only worked with samples allocated by numbers. For other experiments the investigator was not blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The single animal experiment (xenograft) included in the manuscript was performed by a company and the investigators were not blinded during the experiment
5. For every figure, are statistical tests justified as appropriate?	yes, reportet in the figure legends
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, we used visual inspection for assessment
Is there an estimate of variation within each group of data?	yes, with the exception of the westernblots
Is the variance similar between the groups that are being statistically compared?	from visual inspection the variance seems to be similar

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	For each antibody the company and catalog number is indicated
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	P493-6 cells were from D. Eick, Helmholz Centre, Munich, Germany U2OS-MycER cells were from D. Murphy, University of Glasgow, Great Britain, Ramos cells used for the xenograft experiment were certified (STR profiling) by the German Biological Resource Center DSMZ, Braunschweig, Germany . The TSC1 deficient and wt MEFs were from Ken Inoki from the University of Michigan, USA. Mycoplasma contamination was tested regularly by a PCR based method.

* 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.	8 weeks old immunodeficient NOD/SCID female mice were used. Mice were provided by the EPO GmbH company that performed the experiment.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The xenograft experiment was performed according to the German Animal Protection Law with permission of the responsible authorities.
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.	confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The protocols for obtaining human tissue samples were performed in accordance to the guidelined from the Institutional review board or Mediacl Ethical committee of the University Medical Center Groningen, The Netherlands and the University Hospital Jena, Germany
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.	There was consent from all subjects that the experiments conformed to the named principles.
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.	There were no restrictions on the availability or on the use of the human samples
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

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

G- Dual use research of concern

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