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MYC-driven inhibition of the glutamate-cysteine ligase promotes glutathione depletion in liver cancer

Brittany Anderton, Roman Camarda, Sanjeev Balakrishnan, Asha Balakrishnan, Rebecca A. Kohnz, Lionel Lim, Kimberley J. Evason, Olga Momcilovic, Klaus Kruttwig, Qiang Huang, Guowang Xu, Daniel K. Nomura, and Andrei Goga

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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 11 August 2016

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

As you will see, all three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data and the conclusions drawn, which need to be addressed. In particular, further proof of the relevance of the link between MYC and glutathione metabolism in a tumor context needs to be provided (point 1 of referee #2). After cross-commenting, referee #1 agreed with the points (1-6) raised by referee #2 and also suggests that these need to be addressed during revision.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

Anderton and colleagues report that MYC inhibits GCLC and causes glutathione depletion in liver cancer. The paper compares metabolite levels with gene expression data. Then, tracing is performed to attempt to identify or confirm pathways identified. Some of these details were hard to follow, since many of the experiments are in supplementary figures. The authors then chose to focus on GCLC and mir18a. But, the mechanistic studies are thin supporting this role. Moreover, the general significance is not entirely clear. The authors provide some suggestion that in human tumors there is a correlation, which is suggestive. The general significance to tumorigenesis is not clear. Moreover, related work has already been published by the authors on Glutathione metabolism and Fatty Acid Metabolism. Overall this paper seems to report data that were part of but not mentioned in these prior published studies. The notion that GCLC may maintain redox balance is an interesting idea, but the studies as presented are rather premature to support this claim. Overall, this paper as it stands would be reasonable to report for the sake or providing the data and the authors' observations to the field. However, to support the authors's proposed conclusions aregoing to require significantly more experimental effort.

Referee #2:

The authors show a clear and interesting connection between MYC expression and gluthatione metabolism, acting through miR-18a. Moreover, they provide convincing evidence that this newly discovered metabolic consequence of MYC activity is dependent on miR-18a. Correlation of miR-18a and gene expression of gluthatione metabolism enzymes with aggressiveness of HCC was shown.

Major comments:

1) The relevance of this newly discovered link between MYC and glutathione metabolism would increase by showing that the modulation of miR-18a or GCLC directly translates into decreased tumor growth or increased tumor sensitivity towards oxidizing chemotherapeutic agents.
2) The authors place great importance in the fact that a western blot showed increased levels of GGT1 in MYC-driven tumors (figure 2E). However, the blot is not clear in showing an increase. In fact, it seems that there is little to no change in MYC-driven tumors. Since the increased levels of GGT1 are a cornerstone to the hypothesis proposed by the authors that there is increased-gamma glutamyl cycling, more convincing western blots should be provided, or the cycling hypothesis revised.
3) Similarly, the authors state that "they found elevated abundances of several gamma-glutamyl aminoacids" and use this as an argument for increased gamma-glutamyl aminoacid cycling. However, based on figure 2C, there are multiple gamma-glutamyl aminoacids that show either no change, or are even decreased in the MYC-driven tumors. This seems to be contradictory to the idea that there is increased cycling. Is there anything known about the specificity of GGT1 that could explain these results? If not it is advised that the authors review their increased cycling hypothesis.
4) A metabolite of central hypothesis in the gluthatione synthesis pathway is gamma-glutamyl cysteine. However, the authors never include any information on the actual levels of this metabolite. It is clear from figure 2G that it is possible to measure it thus the authors should be able to provide information about the levels of gamma-glutamyl cysteine in control tissue and MYC-drive tumors.
5) For several western blots Ponceau staining is used as a loading control. This however is not sufficient and loading controls such as actin should be shown instead.
6) The authors' model allows, upon the administration of doxycycline to the mice, to decrease MYC expression. The authors only employ this reversion briefly in figure 2E to show that GCLC levels return to normal. It would be interesting, at the very least, to also understand how the levels of the other enzymes in the glutathione synthesis pathway are changed upon decreased MYC expression as well as to determine if, after doxycycline administration, the levels of gluthathione are normalized, as this would provide convincing evidence for the hypothesis of the authors.

Minor comments:

1) On page 10, 5th line from the bottom, figure 5F should be replaced with 4F
2) On page 12, 6th line from the top, the authors reference figure 6, which does not exist.
3) The authors should provide clearer information on how the doxycycline was administered to the mice.
4) When analyzing the previous existing data sets created by Huang et al. the authors claim they replaced "missing values (...) with the minimum values across all samples". The consequence of this replacement is unclear (i.e. are the data altered depending on whether minimum values were used or not?). In general it would seem more correct to simply not replace missing data.
5) Figures 2B and 2F should be combined.
6) In figure 5, the authors put great emphasis on miR-23a. However, this miR is not measured in supplemental figure 9. More information on the levels of miR-23a should be provided
7) Overall, most of the figures lack statistical significance information (see figure 2G and most of the supplementary figures). This should be corrected.

Referee #3:

This manuscript by Anderton et al. describes a thorough and detailed description of mechanisms leading to novel MYC-dependent regulation of glutathione metabolism. The study is well-conceived and the data support the authors' conclusions. I have no major criticisms, although it would have been interesting to see whether NRF2 status correlates with high MYC or AFP expression in HCC.

Minor points:

1. page 10, bottom: I believe that reference to Figure 5F should be changed to 4F. Further, in Supplemental Figure 10A-B, the correlation with GSH levels is with MYC not with AFP as written.
2. page 12, top: Reference is made to Figure 6, but there is no Figure 6 included in the manuscript. In a related point, I think it would be useful in Figure 5 to list some of the other pathways that may utilize glutamate, instead of using the uninformative "other pathway" designation shown in the figure.

1st Revision - authors' response 27 October 2016

Editor's Summary: As you will see, all three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data and the conclusions drawn, which need to be addressed. In particular, further proof of the relevance of the link between MYC and glutathione metabolism in a tumor context needs to be provided (point 1 of referee #2). After cross-commenting, referee #1 agreed with the points (1-6) raised by referee #2 and also suggests that these need to be addressed during revision.

Response: *We have addressed points #1-6 raised by referee #2, as well as the points raised by referees #1 and #3. Notably, we have included new data suggesting that primary MYC-driven liver tumors are uniquely sensitive to acute delivery of a potent oxidant, diquat. We discuss these findings in point #1 of referee #2 below. The corresponding data are now found in* ***Figure 5*** *of the revised manuscript.*

Referee #1:

Anderton and colleagues report that MYC inhibits GCLC and causes glutathione depletion in liver cancer. The paper compares metabolite levels with gene expression data. Then, tracing is performed to attempt to identify or confirm pathways identified. Some of these details were hard to follow, since many of the experiments are in supplementary figures. The authors then chose to focus on GCLC and mir18a.

***Response**: To clarify, we first compared metabolite levels with gene expression data and identified 6 significantly altered metabolic pathways in MYC-driven tumors. Of the 6 pathways, glutathione was the most significantly altered at the transcript level. We performed enzymatic assays and isotopic tracing of 13C-glutamine to confirm that glutathione is depleted and glutathione synthesis is impaired in tumors, respectively. After confirming that glutathione synthesis is impaired in tumors by isotopic tracing, we focused on depletion of GCLC, the rate-limiting enzyme of glutathione synthesis, and the mechanism of its inhibition via miR-18a. In the revised manuscript we have attempted to clarify the workflow (as summarized above) making this clearer in the results section. Unfortunately, because of space limitations, much of the analysis remains in the Expanded View figures, nevertheless we have strived to improve the clarity of our results.*

But, the mechanistic studies are thin supporting this role. Moreover, the general significance is not entirely clear. The authors provide some suggestion that in human tumors there is a correlation, which is suggestive. The general significance to tumorigenesis is not clear. Moreover, related work has already been published by the authors on Glutathione metabolism and Fatty Acid Metabolism. Overall this paper seems to report data that were part of but not mentioned in these prior published studies.

***Response**: We have now provided more evidence of the general significance of our findings to tumor survival under oxidative stress conditions. Please see our response to Reviewer #2's first comment below. Our lab has not previously published on the topic of glutathione or glutathione synthesis, in any tumor models. The report on fatty acid metabolism that we recently reported was specific to a subtype of breast cancer and no experiments in that report were performed in models of liver cancer. Furthermore, our prior work and that of others' (see Yuneva et al., Cell Metabolism 2012) indicate that tumor metabolism is both oncogene- and tissue- specific. Thus, we argue that the data presented in this paper provide novel insight into the metabolism of liver cancers with high MYC expression.*

The notion that GCLC may maintain redox balance is an interesting idea, but the studies as presented are rather premature to support this claim. Overall, this paper as it stands would be reasonable to report for the sake or providing the data and the authors' observations to the field. However, to support the authors' proposed conclusions are going to require significantly more experimental effort.

***Response:** We provide new data showing that MYC-driven liver tumors, which have depleted GSH, show increased sensitivity in vivo to a potent oxidant, diquat (please see our response to Referee #2, point #1). It is widely accepted that GSH is a major cellular antioxidant produced primarily in hepatocytes. Furthermore, GCLC is well established as the rate-limiting enzyme of GSH synthesis. Our work implicates a new role for MYC in regulation of GSH via a microRNA. We have changed our language in the revised text to make it appropriate for the data we show. A new **Figure 5** that incorporates in vivo analysis of tumor response to oxidative stress is now provided in the revised manuscript.*

Referee #2:

The authors show a clear and interesting connection between MYC expression and glutathione metabolism, acting through miR-18a. Moreover, they provide convincing evidence that this newly discovered metabolic consequence of MYC activity is dependent on miR-18a. Correlation of miR-18a and gene expression of glutathione metabolism enzymes with aggressiveness of HCC was shown.

***Response**: We thank Referee #2 for appreciation of the novelty of our findings and their link to human HCC.*

Major comments:

1) The relevance of this newly discovered link between MYC and glutathione metabolism would increase by showing that the modulation of miR-18a or GCLC directly translates into decreased tumor growth or increased tumor sensitivity towards oxidizing chemotherapeutic agents.

***Response**: In the revised manuscript, we have included new data (see **Figure 5**) in which we treat MYC driven tumors that have low GCLC expression with a potent oxidant, diquat. We find that such treatment leads to loss of tumor cellularity, in MYC tumor versus adjacent non-tumor tissues. We also find increased cell death in tumor tissue (indicated by increased TUNEL staining), but not adjacent non-tumor tissue following diquat treatment, consistent with the diminished GSH present in tumors. Finally, following diquat treatment, there is diminished MYC expression in the remaining tumor cells. Taken together, these new data provide strong additional mechanistic support linking suppression of GCLC and hence GSH depletion via MYC to sensitivity to exogenous oxidative stress. The new data and description are now added to the manuscript and can be found in **Figure 5**.*

2) The authors place great importance in the fact that a western blot showed increased levels of GGT1 in MYC-driven tumors (figure 2E). However, the blot is not clear in showing an increase. In fact, it seems that there is little to no change in MYC-driven tumors. Since the increased levels of GGT1 are a cornerstone to the hypothesis proposed by the authors that there is increased-gamma glutamyl cycling, more convincing western blots should be provided, or the cycling hypothesis revised.

***Response:** To address this comment, we have repeated the Western Blot in question. We again found a very small relative expression difference of GGT1 in tumor and non-tumor samples. We have changed the relevant language in the paper to reflect that we observe a very small, but statistically significant, difference in GGT1 protein expression in tumors relative to non-tumors. We have also revised our gamma-glutamyl cycling hypothesis in the text, as recommended by Referee 2 (see results and discussion).*

3) Similarly, the authors state that "they found elevated abundances of several gamma-glutamyl aminoacids" and use this as an argument for increased gamma-glutamyl aminoacid cycling. However, based on figure 2C, there are multiple gamma-glutamyl aminoacids that show either no change, or are even decreased in the MYC-driven tumors. This seems to be contradictory to the idea that there is increased cycling. Is there anything known about the specificity of GGT1 that could explain these results? If not it is advised that the authors review their increased cycling hypothesis.

***Response**: We thank Referee #2 for pointing out the discrepancies related to our original hypothesis of increased gamma-glutamyl cycling in MYC-driven tumors. To our knowledge, GGT1 catalyzes*

transpeptidation reactions in the presence of high concentrations of amino acids. However, we do not have evidence for relative levels of free amino acids in the tumors to explain the differences observed. We have revised our hypothesis that increased gamma-glutamyl cycling occurs to reflect the Western Blot data and the inconsistency of changes in levels of gamma-glutamyl amino acids observed. The main focus of the manuscript remains the role of GCLC in glutathione biosynthesis, and we now de-emphasize the potential roles of gamma-glutamyl cycling. These changes do not alter our overall findings and the importance of GCLC regulation by MYC.

4) A metabolite of central hypothesis in the glutathione synthesis pathway is gamma-glutamyl cysteine. However, the authors never include any information on the actual levels of this metabolite. It is clear from figure 2G that it is possible to measure it thus the authors should be able to provide information about the levels of gamma-glutamyl cysteine in control tissue and MYC-drive tumors.

Response: *Unfortunately, gamma-glutamyl cysteine was not reported in our initial metabolite profiling dataset. However, we have performed 12C metabolite profiling on MYC-driven liver tumors and adjacent non-tumor control tissue. We find that gamma-glutamyl cysteine is significantly depleted in tumors relative to non-tumor controls, further supporting our hypothesis that GCLC activity is diminished in MYC-driven liver tumors. We have included this new data in* ***Figure 2C****.*

5) For several western blots Ponceau staining is used as a loading control. This however is not sufficient and loading controls such as actin should be shown instead.

Response: *One challenge with studying primary tumors versus non-tumor tissues is that the size and morphology of tumor cells is very different from non-tumor tissues. For example, MYC-driven liver tumor cells are smaller and with a higher nuclear to cytoplasmic ratio (see* ***Figure 1A****). We have found that several commonly used loading controls, including beta-tubulin and beta-actin, exhibit dramatically different protein expression in tumor versus non-tumor tissues (please see representative blots in* ***Response Figure 1A-B [data not included in review process file]****). Likewise, we felt that another common loading control, GAPDH, is not appropriate for our study because MYC is a known regulator of multiple metabolic (and, in particular, glycolytic) enzymes. We have thus chosen to represent Ponceau staining as a total protein loading control, the same as our colleagues did when they published protein expression data from the same tumor model previously (please see Yuneva et al., Cell Metabolism 2012).*

6) The authors' model allows, upon the administration of doxycycline to the mice, to decrease MYC expression. The authors only employ this reversion briefly in figure 2E to show that GCLC levels return to normal. It would be interesting, at the very least, to also understand how the levels of the other enzymes in the glutathione synthesis pathway are changed upon decreased MYC expression as well as to determine if, after doxycycline administration, the levels of gluthathione are normalized, as this would provide convincing evidence for the hypothesis of the authors.

Response: *Because regulation of GCLC is a central focus of this manuscript, we evaluated its protein expression upon tumor regression and found that its expression is MYC-dependent (****Figure 2D****). We further provide evidence for specific regulation of GCLC by miR-18a, a miRNA that is directly transcriptionally regulated by MYC (He et al., Nature 2005). In the current study, we have not explored if other enzymes related to glutathione synthesis are also regulated by MYC (by either direct transcriptional regulation or via a miRNA). For example, there is no data to suggest that miR-18a is also predicted to regulate other glutathione synthesis pathway genes. Thus we believe that including the expression of the other proteins following tumor regression does not fit the scope of this paper, which focuses on the miR-18a-dependent regulation of GCLC by MYC.*

However, we agree that it would be interesting to know if other glutathione pathway genes exhibit MYC-dependent or -independent regulation. We have extracted data from a recent study that evaluated MYC-dependent regulation of the mouse transcriptome in the same tumor model utilized in our study (Kress et al, Cancer Research 2016). This information is now included in ***Table S3****, to complement the protein expression we see in MYC liver tumors versus non-tumor controls. To summarize, Ggt1 and G6pdx were found to have MYC-independent transcriptional upregulation; Gls was found to have MYC-dependent transcriptional upregulation; and Gls2 was found to have MYC-independent downregulation. The remaining proteins (Gss, Glrx5, Gsr) were not found to have any MYC-dependent transcriptional regulation.*

To address the reviewer's second question of whether glutathione levels are normalized upon tumor regression, we have now included data showing that in tumors regressed for 72h, there is no statistically significant difference in total glutathione (GSH + GSSG) abundance, as compared to non-tumor control tissue. This suggests that the levels of glutathione return to normal non-tumor levels upon tumor regression. This new data is now included in ***Figure EV4B****.*

Minor comments:

1) On page 10, 5th line from the bottom, figure 5F should be replaced with 4F

Response*: Thank you, we have fixed this error.*

2) On page 12, 6th line from the top, the authors reference figure 6, which does not exist.

Response*: Thank you, we have fixed this error.*

3) The authors should provide clearer information on how the doxycycline was administered to the mice.

Response: *We have included more detailed information in the Materials and Methods section, noting that doxycycline was administered in the mouse chow (200 mg/kg), which was consumed ad libitum by the mice. We have also provided detailed information about how regression was induced, by placing the mice back on doxycycline chow, fed ad libitum to inhibit MYC expression.*

4) When analyzing the previous existing data sets created by Huang et al. the authors claim they replaced "missing values (...) with the minimum values across all samples". The consequence of this replacement is unclear (i.e. are the data altered depending on whether minimum values were used or not?). In general it would seem more correct to simply not replace missing data.

Response: *The process of imputing minimum values is used to address limitations in the sensitivity of the instruments used to measure metabolite abundance. This process assumes that the measurements in question (the missing values) fell below the detection range of the instrument. Rather than filling a blank value with zero, the minimum observed value for each variable in the given dataset is imputed. This is a common practice used in metabolomics analyses to keep the variation within a dataset smaller, while accounting for the full range of values that the instrument is able to accurately detect (see Chen et al., Plos One 2015; Adams et al., Osteoarthritis Cartilage 2012; Menon et al., Reproductive Sciences 2014; Brown et al., Cancer & Metabolism 2016).*

5) Figures 2B and 2F should be combined.

Response*: Thank you, we have combined the schematics into one,* ***Figure 2B****.*

6) In figure 5, the authors put great emphasis on miR-23a. However, this miR is not measured in supplemental figure 9. More information on the levels of miR-23a should be provided

Response: *Thank you for pointing out this inconsistency. Reviewer #2 is right that we do not include data for miR-23a in this study. We intended to use the schematic figure to reference conclusions from a separate study (Gao et al., Nature 2009) whose focus is on miR-23a/b. We sought to incorporate this prior work into a more inclusive model of how MYC-regulated miRNAs (ie miR-18a and miR-23a/b) alter glutamine metabolism. However, based on the reviewer's comment we have decided not to include miR-23a in our summary schematic, as we do not provide direct data for its expression in our analysis. We have thus removed it from the summary figure (**Figure 6** in the revised manuscript).*

7) Overall, most of the figures lack statistical significance information (see figure 2G and most of the supplementary figures). This should be corrected.

Response: *Thank you for pointing this out. We have added statistical significance information where appropriate, including Figure 2G (now **Figure 2E**) and the Supplementary figures, including **Figure EV1B-C**. Statistical information is also provided in all figure legends. Please note that for some figures, such as **Supplemental Figures 1-6**, all variables shown are statistically significantly different between tumor and non-tumor. This is noted in the appropriate figure legends.*

Referee #3:

This manuscript by Anderton et al. describes a thorough and detailed description of mechanisms leading to novel MYC-dependent regulation of glutathione metabolism. The study is well conceived and the data support the authors' conclusions. I have no major criticisms, although it would have been interesting to see whether NRF2 status correlates with high MYC or AFP expression in HCC.

Response: *We thank Reviewer #3 for their appreciation of our work. We agree that NRF2 status with regard to MYC in HCC would be interesting. Our preliminary Western Blots did not indicate changes in the level of NRF2 protein expression in tumor versus nontumor tissue in the mouse model (please refer to **Figure 2A** in the Response figures **[data not included in review process file]**). Further, we did not observe changes in Nfe2l2 or Keap1 (post-translational regulator of NRF2) mRNA in tumor compared to nontumor tissues (**Figure 2B** in the Response figures **[data not included in review process file]**). We saw no evident correlation between aberrations in c-Myc, Keap1, or Nfe2l2 in two distinct human HCC datasets available on the Cancer Bioportal website (**Figures 2C-D** in the Response **[data not included in review process file]**). Finally, a study evaluating MYC-dependent regulation of the mouse transcriptome in the same tumor model utilized in our study (Kress et al, Cancer Research 2016) did not identify either MYC-dependent (ie, transcriptional) or -independent (ie, post-transcriptional) regulation of either Nfe2l2 or Keap1. To summarize, there is no compelling data that NRF2 (Nfe2l2) or Keap1 expression is altered in MYC-driven liver cancers. We have thus provided this data for the reviewer and editor (see attached data that accompanies this response below **[data not included in review process file]**) but have chosen not to include this in the manuscript. However, we would be happy to include this in the Expanded View data if the editor and/or Referee felt that this was important.*

Minor points:

1. Page 10, bottom: I believe that reference to Figure 5F should be changed to 4F. Further, in Supplemental Figure 10A-B, the correlation with GSH levels is with MYC not with AFP as written.

***Response**: Thank you for pointing out these errors. We have addressed these in the relevant text. For **Figure 10A-B**, the correlation was shown as intended. AFP is a marker of aggressive disease that correlates with MYC. We have clarified the language in the manuscript text to make the connection between MYC and AFP, and the relevance of including the data in Supplemental Figure 10A-B (now **Figure EV5A-B**), clearer.*

2. Page 12, top: Reference is made to Figure 6, but there is no Figure 6 included in the manuscript. In a related point, I think it would be useful in Figure 5 to list some of the other pathways that may utilize glutamate, instead of using the uninformative "other pathway" designation shown in the figure.

***Response**: Thank you for pointing out this error. We have corrected that reference. We have also updated the summary **Figure 6** to be more informative.*

2nd Editorial Decision 07 December 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study that you will find enclosed below. As you will see, two referees now support the publication of your manuscript in EMBO reports, whereas referee #1 is still critical, in particular regarding the suggested mechanism. Nevertheless, after cross commenting with the other referees, and as EMBO reports emphasizes novel functional over detailed mechanistic insight, we think that the manuscript is now suitable for publication. However, I have several editorial requests that need to be addressed during a final revision.

Please submit single high-resolution versions of all main and EV figures in TIFF or EPS format. In Fig. 2D the labeling is slightly messed up (2x "Tumo" with the "r" below and obscured by the image; 1x "ctrl" obscured by the image). Please adjust this.

The source data needs to be separated and also submitted as one PDF file per figure or per figure panel.

Please add a running title, up to five key words, a conflict of interest statement and the author contributions to the main manuscript text. The abstract is currently too long. Please shorten it to not more than 175 words.

Please add contents to the database link in the methods section (https://github.com/romancamarda/myc_GCLC_study). In the checklist you indicate that "metabolomic data will be deposited as per editor's recommendation". Please do or explain.

Further, for a short report the manuscript is rather long. Usually, the main text (without M&M and the references) should have 25000 characters (with spaces), at maximum 27000. I therefore need to ask you to shorten the paper. We also ask to combine the results section and the discussion for a short report. The other possibility would be to publish this as a research article. See also: http://embor.embopress.org/authorguide#researcharticleguide

The material and methods section is currently very long and detailed. Significant shortening would be very welcome.

The EV figure legends are inconsistently formatted (both compared to the other main figures and to each other). Please standardize formatting of these legends, in particular how the panel letters are displayed and highlighted (maybe just like in the legends of the main figures).

It seems the figure legend for Fig. 2 was duplicated, whereas the legend to Fig. 3 is missing. Please correct this.

Finally, please remove the Appendix figure legends from the main manuscript text (as they are contained in the Appendix).

REFEREE REPORTS

Referee #1:

The authors have made efforts to delineate further the link between MYC and GSH by adding two new pertinent data Figure 5 and Table S3. This provides some suggestion of a mechanism but only indirectly and lacks a causal demonstration. The general significance of the findings are still compromised.

Figure 5 shows treating MYC driven tumors that have low GCLC expression with a potent oxidant leads to cell death by TUNEL staining. This is suggestive of a role.

Table S3 Microarray analyses was done to show GSH metabolism regulation by MYC. But, this analysis mostly shows many MYC-independent GSH genes. This at least makes it seem to make more sense as to why they chose to look into GCLC. But, the data are not entirely supportive.

Referee #2:

The authors have addressed all my concerns with adequate experiments or explanations.

Referee #3:

The authors have sufficiently addressed the major points raised by the reviewers. As such, the revised manuscript is significantly improved and is not in need of further revision.

2nd Revision - authors' response 20 December 2016

Thank you for your review of our manuscript entitled "***MYC Inhibits GCLC and Contributes to Glutathione Depletion in Liver Cancer***" by Anderton et al. for consideration in ***EMBO Reports***. We were glad to receive your response that the manuscript is now acceptable for publication. We have now sought to address the remaining issues outlined below, and now submit a final manuscript that addresses these issues.

- Manuscript length: Because of the length of the manuscript and the amount of data contained, per your suggestion we have now changed the format of the manuscript to a 'Research Article'. We have also further shortened and clarified the materials and methods section, and have moved some of the methods to a supplemental section.

- We have now addressed the issues with figure formatting, small errors in figure legends, and other formatting issues. We now provide high resolution TIFFs for submission.

- The 'code availability' has been correctly linked to the available code in GitHub.

- Since there is no centralized repository for metabolomics data, analogous to GEO, we have attached an excel sheet that contains the primary metabolomics data which is now uploaded as file DatasetEV1 for the manuscript.

I believe the novel MYC-regulated metabolic pathway we describe in this manuscript will be of broad interest to readers of ***EMBO Reports*** and to those studying MYC function, cancer metabolism, miRNAs and cancer, and aggressive subtypes of HCC. Thank you for your support of our work.

3rd Editorial Decision 02 January 2017

Thank you for the submission of your revised manuscript to our editorial offices. I apologize for the delay in getting back to you, which is due to the Christmas holidays during which our editorial office was closed. While going through your manuscript we noted a couple of further minor issues that need to be adjusted before we can proceed with acceptance.

Please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Please also remove the summary and the key findings from the manuscript main text. I have saved these and will provide them to the publisher upon acceptance/export.

It is our policy that all material and methods information needs to be contained in the main text. Therefore, please add back the M&M part now in the appendix to the main material and methods section. As you decided that your manuscript would be published as an article (please select "article" as manuscript type when you re-submit), the length is not an issue. Please also remove the statement that further material and methods information is contained in the appendix from the main text.

Then, please name the appendix "Appendix" when you upload the modified file and add page numbers to the TOC and the appendix pdf.

Please provide up to five key words and add them below the running title in the main text.

Finally, I could not find a reference in the text regarding Dataset 1. Please add a call out for this in the main text. Please also provide a separate word or text file with a title for this dataset and a short legend explaining what it contains.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision - authors' response 08 January 2017

The authors made the requested changes and resubmitted the final version of the manuscript.

4th Editorial Decision 13 January 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andrei Goga
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43068V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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.

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

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

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
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http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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?	Please see 'Statistical Analysis' section of Materials and Methods
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Please see 'Statistical Analysis' section of Materials and Methods
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Please see 'Statistical Analysis' section of Materials and Methods
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.	Please see 'Metabolic Analyses,' 'Locked-Nucleic Acid (LNA) Experiments,' 'Diquat Experiments,' 'Statistical Analyses' sections of Materials and Methods
For animal studies, include a statement about randomization even if no randomization was used.	Please see 'Statistical Analysis' section of Materials and Methods
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.	Please see 'Statistical Analysis' and 'Histological Analyses of Murine Liver Tumor Samples' sections of Materials and Methods
4.b. For animal studies, include a statement about blinding even if no blinding was done	Please see 'Statistical Analysis' and 'Histological Analyses of Murine Liver Tumor Samples' sections of Materials and Methods
5. For every figure, are statistical tests justified as appropriate?	Please refer to Figure Legends and 'Statistical Analysis' section of Materials and Methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Please see 'Statistical Analysis' section of Materials and Methods
Is there an estimate of variation within each group of data?	Please see 'Statistical Analysis' section of Materials and Methods
Is the variance similar between the groups that are being statistically compared?	Please see 'Statistical Analysis' section of Materials and Methods

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Please see 'Protein Preparation and Western Blot Analysis' section of Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Please see 'Murine Liver Tumor Cell Lines' section of Materials and Methods

* 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.	Please see 'Ethics Statement,' 'LT2-MYC Tumor Generation and Regression,' 'Metabolic Analyses,' 'Locked-Nucleic Acid (LNA) Experiments,' and 'Diquat Experiments' sections of Materials and Methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Please see 'Ethics Statement' section of Materials and Methods
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.	Please see 'Ethics Statement' section of Materials and Methods

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Please see 'Data Availability' section of Materials and Methods
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).	Metabolomic profiling data available as Dataset 1 with the manuscript
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Please see 'Data Availability' section of Materials and Methods
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Please see 'Code Availability' section of Materials and Methods

G- Dual use research of concern

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