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Functional interplay between c-Myc and Max in B lymphocyte differentiation

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1st Editorial Decision 26th Jan 18

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

As you will see, all referees think the manuscript is of interest, but requires major revision to allow publication in EMBO reports. All referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. We feel, however, that in particular the points regarding the deletion (and expression) of Myc and Max need to be addressed experimentally (point 2 and 4 of referee #2, major concerns of referee #4), as well as the concerns about GC development (referee #4).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a 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 COMMENTS

Referee #1:

The manuscript by Perez-Olivares generates and characterizes mice in which Max, Myc or both

Max and Myc are deleted in developing and mature B cells. The strength of the manuscript is that it provides what appears to be the first description of cells with conditional deletion of Max and does so in a highly relevant setting. The weakness of the manuscript is that the discussion/interpretation of results is superficial, often confusing and in places not well-thoughtout. This is an important study that deserves more critical evaluation/interpretation of the data to draw out its significance.

1. Abstract.
A. It would help readers, particularly those not B cell aficionados, to provide some kind of description of what is meant by hyperactive state.
B. The last sentence is circular and extremely confusing.

2. Introduction
A. There is redundancy and discord in the first paragraph.

B. The Tu reference in paragraph 2 is focused on Myc-interacting factors, not on Max-interacting factors as the sentence refers to. Appropriate review articles on the later exist and should be used. Same with the Tu reference on page 6, 9 and 10.

C. Loss of Max in pheochromocytomas and other neuroendocrine tumors is highly relevant to the work and should be mentioned in the introduction and/or in the discussion. Providing such context should help in understanding the significance of findings in this work.

D. In general the writing is somewhat disjointed.

3. Results/Discussion.
A. Figure 1I, J. Its not clear why DKO-mb1 cells were not analysed (like in the other analyses in Figure 1). Such information would provide a more complete analysis.

B. The effect of loss of Myc on CDC7 expression is very interesting. Is a transcriptional mechanism responsible? If so, was this dramatic downregulation picked up in the RNA-seq experiments? More information (and potentially disscussion) concerning whether the stunning absence of CDC7 is due to it being a direct or indirect target of Myc (or is due to an non-transcriptional phenomenon) and how loss of Max appears to rescue its expression is warranted.

C. For gene expression profiles, the number of differentially expressed genes mentioned in the text should be 2,604, 3,313 and 2,559 not 2.604, 3.313 and 2.559. And later 1,921 not 1.921.

D. Much of the writing in figure 4 is too small to see.

E. There is no specific explanation on page 8 for why the conclusion that "the lack of c-Myc, Max or both drives B cells to a hyperactive state." This needs to be more explicitly connected to previous sentence - preferably with relevant references.

F. In general no meaningful context is provided for the changes observed by RNA-seq. For example, how does the data compare to other analyses of cells that do not have Myc? It is not clear what might be novel and what is consistent with other studies. Has Myc been previously implicated in Parkinson's or Huntingson's disease and does this study do so?

G. An independent confirmation of specific examples of gene expression changes (perhaps ones identified in the gene pathways affected (Figure 4G)) by qPCR would be helpful. Much is know about Myc and its transcriptome. Have any of these pathways been implicated as regulatory nodes for Myc or Max? If so, mention, if not, please provide an explanation.

H. Page 9 - "In the absence of Myc, Max plays an inhibitory role" etc. This statement lacks any meaningful context and needs to be explained in an coherent, reference supported fashion.

I. Page 9 - "c-Myc has a marginal role in primary B cells". This is an incorrect statement - right?

J. Typos in text and Fig Legends - additional proofreading is needed.

Referee #2:

Building on previous studies of the impact of loss of MYC, Perez-Olivares et al have used conditional gene deletion in mice to investigate the impact on B lymphoid ontogeny of loss of MAX, either alone or together with loss of MYC. The major findings reported are as follows:

1. By crossing mb1-Cre and floxed Max mice, together with a rosa26gfp/gfp reporter, they found that although mice lacking MAX, or both MYC and MAX, had significantly reduced numbers of total B lymphoid cells in their bone marrow and spleen, those cells were able to differentiate relatively normally to the Ig-positive B cell stage, both in vivo and in vitro. These results stand in contrast to a previous study from this lab (Vallespinos et al 2011) showing that loss of MYC resulted in a developmental block at the pre-B cell stage. Analysis of pro- and pre-B cells taken from the MaxKO-mb-1 and MaxMycDKO-mb-1 mice suggested that loss of MAX resulted in a slight reduction in cell cycling but not apoptosis.

2. By crossing with CD19-Cre mice, they showed that both MYC and MAX are required to achieve normal levels of class switching and production of Antibody-secreting Cells (ASC) and that MAX was necessary for the generation of germinal centres, as previously shown for MYC. Using cultured B220+IgM+ spleen cells (and cell tracking dye, they showed that lack of MAX, or both MAX and MYC, hindered proliferation but did not completely block it, whereas lack of MYC prevented proliferation. These findings were substantiated by DNA replication assays. Loss of MYC correlated with loss of CDC7, providing a plausible mechanisms for dramatic impact of loss of MYC on replication capacity.

3. RNA-Seq data obtained from mature B lymphocytes of MaxKO-CD19Cre, MycKO-CS19Cre and control HET-CD19Cre mice lead the authors to conclude that lack of MYC, MAX or both drives B cells to a hyperactive state.

This genetic study will be of interest to the many scientists studying the network of MYC-related transcription factors, although the mixed genetic background (see below) is a major negative factor. The data are consistent with the widely held view that MAX is an obligate partner of MYC in regulating transcription. A key finding is that the MYC/MAX heterodimer facilitates but is not absolutely essential for differentiation and DNA replication in B lymphoid cells. The conclusion that lack of MYC, MAX or both drives B cells to a hyperactive state appears overstated (see below) and not warranted in the Abstract.

Major and minor points that should be addressed by the authors are indicated below.

Major points
1. The mixed genetic background (see Materials and Methods) materially detracts from this study as differences in the expression of genes other than Myc or Max may have impacted upon the phenotypes. This possibility should be acknowledged as a caveat to conclusions drawn about differences in phenotype and gene expression.

2. For each Cre transgene, efficiency of Max (and Myc) deletion (GFP-positivity) should be indicated and also assessed by PCR and western blot analysis.

3. The text should explain the reason for switching from Mb1-Cre to CD19 Cre mice subsequent to the analyses shown in Figure 1.

4. Figure 1
- - Data for MycKO should have been included to compare with that of the MaxKO and DKO mice. It is insufficient to rely on historical data.
- - Indicate the age and number of independent mice of each genotype that were immunophenotyped. Were they sex matched?
- - 1F Define what is meant by total B cells - B220+ or B220+Ig+ ? There appears to be either a calculation error or the labelling is too poor in Fig1F, G, H to interpret. Fig 1F shows a <2x decrease

in BM and spleen B cells in the DKO, yet the more precise data in panels G and H show much stronger deceases in DKO (>10x). Moreover if one adds up the numbers of the Bcell fractions in panel 1G or 1H they are much lower than the total B cell numbers in panel F.
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5. Figure 2
- Indicate the statistical significance for MaxKO-CD19 and DKO-CD19 in comparison to both HET-CD19 and MycKO-CD19.

6. Conclusion on page 7 end of 1st para should presumably be 'However, in the absence of Max, Myc or both, B cells in CD19Cre mice can still generate ASC and perform CSR.'
7. Figure 4. The conclusion that loss of Myc/Max "drives cells to a hyperactive state" seems dubious. The KO cells do not proliferate well, are smaller (decrease FSC in fig3D) and processes such as ribosomal pathway, translation, oxidative phosphorylation and metabolism are down-regulated. The authors do show that some signalling pathways might be up in KOs but this might simply be due to the lack of differentiation compared to the Het control.

8. Figure 3.
-legend states that data in A are representative of at least 3 independent experiments. How many mice were used in each arm of each experiment? Gating of cell cycle analysis in 3E is strange. Not sure how they define the S phase as the dashed line seems too high. The gating of the same type of data in Figure 1I is much better and very different from 3E.

Minor points
1. Vallespinos et al is missing from the reference list.

2. Flow cytometry is two words rather than one.

3. The authors should comment on whether N-Myc or L-myc are expressed in this system and possibly complicate the interpretation. They have this in their RNAseq data

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Referee #3:

In this study, Perez-Olivares et al. have conducted conditional knockout study about c-Myc and Max in primary B lymphocytes. Previously this group reported that c-Myc is crucially important for the development in B lymphocyte. However, unexpectedly, the authors encountered much milder phenotype with Max knockout B cells, although c-Myc present in Max-knockout B cells is nonfunctional as a transcriptional factor in the absence of Max expression. Then, the authors generated c-Myc/Max double knockout B cells and again, the authors observed much milder phenotypes compared to B cells which are null only for c-Myc. Based on their findings the authors concluded that c-MYC/MAX complex is intrinsically dispensable for maturation of B cells. Furthermore, the authors speculate that severe phenotypes observed with c-Myc-null B cells attribute to MAX present in those cells.
I consider that their data merit publication in the journal, since their data have changed the concept of role of c-Myc/MAX complex in this biological process, although this manuscript lack data of the molecular bases of MAX function leading detrimental phenotype in c-Myc null B lymphocytes.

However, I ask the authors to conduct the following experiments to address my concern. I felt weird that the authors did not show data obtained form cMyc-KO-mb1 in this manuscript, although they showed such data previously. I consider that the authors should simply show phenotypic differences between cMyc-KO-mb1 and Max-KO-mb1 by providing both in Figure 1 of this manuscript.

In addition, I strongly recommend to change the title to the one that represent their findings clearly.

Ideally, it is better to explore whether MAX requires additional factor such as Mad to exert detrimental phenotype of cMyc-KO B lymphocytes.

Referee #4:

In this report the authors generate a conditional Max knock out mouse and examine the contribution of Max to Myc dependent roles in B cell development in vivo and B cell differentiation and class switching in vitro. The authors perform extensive breedings to obtain floxed Myc, Max and Myc/Max alleles with B cell specific Cre strains (Mb1, CD19) in combination with an R26-YFPflSTOP reporter. Thus the authors have potentially interesting tools in their hands, but unfortunately these tools are not used to their full potential. The most critical problem is the lack of evidence of Max and Myc protein (and mRNA) deletion throughout the paper. The authors use the expression of the YFP reporter (R26-STOPfl-YFP) as an indicator of efficient Myc/Max deletion. However, the YFP reporter and the Max/Myc genes are located on independent alleles, and in conditions of a possible strong counterselection of Myc/Max deleted cells the expression of YFP does not provide sufficient evidence for their deletion. Moreover, the Max conditional knock out is a novel, previously unpublished mouse model in which the last two exons (4 and 5) are flanked by loxP sites. It is thus important to show what is the nature of Max protein expression upon Cre mediated deletion (a truncated, unstable, Myc binding protein?).

Major concerns:
Figure 1-4: Lack of Myc/Max deletion efficiency data on protein and mRNA level.
Figure 1. B-J: Lack of MycKO-mb1 controls.
Figure 1. I-J: Lack of Myc/MaxDKO-mb1.

Figure 2. A-H: Lack of deletion efficiency data in d3 B cell cultures and sorted ASC and IgG1+ B cells. Given the very low efficiency of plasma cell differentiation and CSR in the mutants, it is absolutely critical to show that the observed cells are indeed Myc/Max knock-outs.
Figure 2. I-J: Methods state that GCs were scored 30 days post immunization. That is an unreasonable time for primary B cell responses. Standard protocol is 10-14 days.

Figure 3. C-D: Bright field images of activated B cell cultures as an indicator of B cell size have very limited if any value. It is difficult to understand what the authors want to conclude from an FSC histogram plot, given the fact that the FSC hi blasting B cells (present in the control around the value of 400K) are completely absent in all KO plots.

Figure 4. E, G: From RNA seq analysis of B cells activated with LPS+IL4 for three days in vitro the authors conclude that B cells lacking Myc and/or Max (whose absence is once again not shown) are in a "hyperactive state". This conclusion based solely on mRNA expression data, seems inappropriate, particularly in view of the comparable levels of the CD69 activation marker before and after activation in Figure 2G.

Supplementary Figure 4. A. Authors claim: "as an internal control for RNA seq, we observed a similar gene expression profile of previously reported genes (Fernandez et al. 2013", Fig 5). However, of 11 highlighted genes, expression of 5 does not correlate with the data of Fernandez et al.

1st Revision - authors' response 15th Jun 18

Response to referees

We thank all the reviewers for their many useful comments, suggestions and concerns that have helped us to greatly improve our manuscript. We have addressed all the points and performed all the requested experiments. Noteworthy, one of the main concerns of the referees was the status of Myc and Max proteins and mRNA in B lymphocytes in the different KO mice. Unexpectedly, we found that c-Myc protein was decreased in MaxKO B lymphocytes despite normal levels of *c-myc* mRNA. This result has helped us enormously to interpret our data. Specifically, we now better understand why MaxKO cells are more similar to DKO than c-MycKO B lymphocytes. Thus, we have taken into account this result and discussed our data within this context. We believe that the Max-dependent mechanism controlling Myc protein levels in mature B lymphocytes deserves further study but is well beyond the scope of this manuscript. Finally, similar points raised by different referees are indicated and have been answered in the first corresponding section.

Response to referee # 1

One of the main concerns raised by Referee#1 was to discuss and interpreted our data in more detailed manner. We have closely followed the referee´s recommendations. All the points were addressed as follows:

1.*Abstract.*

A. It would help readers, particularly those not B cell aficionados, to provide some kind of description of what is meant by hyperactive state.

The term "hyperactive" is clearly ambiguous and has raised similar concerns in referee #2 (point 7), and referee #4 (point 7). We have addressed this issue in point 3E. Consequently, we have eliminated the sentence containing the term hyperactive from the abstract.

B. The last sentence is circular and extremely confusing.

We have modified the sentence and incorporated in the previous one as "Our data suggest that c-Myc/Max heterodimers are not essential for the initiation of certain biological processes in B lymphocytes. Rather, Myc/Max are necessary for fine-tuning the initial response in these cells after activation".

2. *Introduction*

A. There is redundancy and discord in the first paragraph.

We have modified the first paragraph eliminating the redundancy "Among these factors members of the Myc family (N-, L- and c-Myc) play a prominent function. "… The Myc proteins are involved in many biological functions such as regulation of cell cycle, differentiation, metabolism or apoptosis [1,2].

B. The Tu reference in paragraph 2 is focused on Myc-interacting factors, not on Max-interacting factors as the sentence refers to. Appropriate review articles on the later exist and should be used. Same with the Tu reference on page 6, 9 and 10.

We apologized for this error and included appropriate references. [3,4]

C. Loss of Max in pheochromocytomas and other neuroendocrine tumors is highly relevant to the work and should be mentioned in the introduction and/or in the discussion. Providing such context should help in understanding the significance of findings in this work.

We have expanded our comments on Max-deficient tumors and included the work of [5] and [6]. These modifications are on page 4.

D. In general the writing is somewhat disjointed.

We have edited and proofreading the text to make it more understandable.

3. *Results/Discussion.*

A. Figure 1I, J. It is not clear why DKO-mb1 cells were not analysed (like in the other analyses in Figure 1). Such information would provide a more complete analysis.

At the time of carrying out these experiments *DKO-mb1* mice had not been generated yet. We have repeated the experiments and included the analyses not only *DKO-mb1* but also *MycKO-mb1* B cells in our studies (Fig. 1B- J). The results confirmed our previous observations and provided more compelling evidence. We have modified the text accordingly on pages 5-7.

B. The effect of loss of Myc on CDC7 expression is very interesting. Is a transcriptional mechanism responsible? If so, was this dramatic downregulation picked up in the RNA-seq experiments? More information (and potentially discussion) concerning whether the stunning absence of CDC7 is due to it being a direct or indirect target of Myc (or is due to a non-transcriptional phenomenon) and how loss of Max appears to rescue its expression is warranted.

We did not find downregulation of *cdc7* in our RNA-Seq data. We also performed qPCR analysis of *cdc7* and found no difference among the different mutant and control mice. This information has been added on page 10 and Fig 5C. The rescue of CDC7 expression in Max KO and DKO lymphocytes is intriguing. Our initial interpretation was that Myc regulated CDC7 expression by non-transcriptional mechanisms without the requirement of Max. However, the reduced levels of c-Myc in MaxKO B lymphocytes helped us to provide a better explanation on pages 10-11. "We speculate that in the absence of Myc and Max B lymphocytes are capable to initiate different functions such as regulation of CDC7. However, both factors are required for fine-tune this function. In the absence of c-Myc, we hypothesize that Max alone or with other members of the Myc network downregulates CDC7 levels in c-MycKO B cells…".

C. For gene expression profiles, the number of differentially expressed genes mentioned in the text should be 2,604, 3,313 and 2,559 not 2.604, 3.313 and 2.559. And later 1,921 not 1.921.

We have corrected this mistake and apologize for it.

D. Much of the writing in figure 4 is too small to see.

We have split up Figure 4 in two (Fig. 4 and 5) to allow a better display.

E. There is no specific explanation on page 8 for why the conclusion that "the lack of c-Myc, Max or both drives B cells to a hyperactive state." This needs to be more explicitly connected to previous sentence - preferably with relevant references.

A similar point was raised by referee #2 (point 7) and #4. Our original idea was that mutant B cells were not capable of "processing" the activation signal, and thus this would lead to a constitutive activation which we called "hyperactive". We clearly overinterpreted our data. Our results showed an upregulation of gene expression of different members of prominent signaling pathways. These data might suggest that signaling could be affected but by no means demonstrate *per se* that these pathways are actually functional conferring the cells an activated state beyond normal levels. As referee #2 mentioned in point 4, there are many biological processes impaired (proliferation, cell size, phosphorylation, ribosome...) or normal levels of surface CD69 (referee#4) are difficult to reconcile with a constant activated state. Based on these arguments, we have eliminated the term "hyperactive state" and rephrase on page 12 "These data suggest that the lack of c-Myc, Max or both drives B cells to upregulation of expression of genes involved in different signalling pathways".

F. In general no meaningful context is provided for the changes observed by RNA-seq. For example, how does the data compare to other analyses of cells that do not have Myc? It is not clear what might be novel and what is consistent with other studies. Has Myc been previously implicated in Parkinson's or Huntington's disease and does this study do so?

As far as we know, our report is the first one to analyze RNA-seq data on primary mature B lymphocytes lacking c-Myc. To address the referee´s concern, we have re-written (page 12) this section and added more information to provide a more comprehensive discussion of our data in comparison with previously reported studies. We have compared our results with those of relevant and closely related studies that supplied raw data to allow bioinformatic analysis. These studies were performed on primary mouse B cells overexpressing Myc [7] and c-Myc-deficient T lymphocytes [8]. Noteworthy, the experimental conditions, such as the stimulus, kinetics or cell type largely differ between reports and thus comparison and interpretation have to be taken with caution. Despite all these considerations, we found similar group of genes and pathways between these studies and our data. This information has been included on page 12 and Fig EV5.

A far as we know, c-Myc has not been involved in Parkinson's or Huntington´s disease and our study does not aim to do so. Bioinformatics analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG) can sometimes overinterpret gene expression analyses by assigning molecular pathways or diseases to a group of genes involved in several molecular pathways. To avoid any misunderstanding, we have changed *"Downregulated genes comprised several pathways that included Parkinson's, Huntington's disease, or*

oxidative phosphorylation (Fig EV4C)" by "*Downregulated genes comprised a variety of molecular pathways" (page 13)*.

G. An independent confirmation of specific examples of gene expression changes (perhaps ones identified in the gene pathways affected (Figure 4G) by qPCR would be helpful. Much is known about Myc and its transcriptome. Have any of these pathways been implicated as regulatory nodes for Myc or Max? If so, mention, if not, please provide an explanation.

We have performed gene expression analyses by qPCR (Fig 5C) of randomly picked genes associated with the different pathways shown in Fig. 5B. All the genes tested confirmed the RNA-seq data. In addition, we have double-checked *max* and *c-myc* expression by qPCR (Fig. 5B). We have further discussed the implication of these pathways in Myc/Max regulation. This information is provided on page 11-13.

H. Page 9 - "In the absence of Myc, Max plays an inhibitory role" etc. This statement lacks any meaningful context and needs to be explained in a coherent, reference supported fashion.

We have modified the text to explain in more detail the meaning "In the absence of Myc, Max plays an inhibitory role" on pages 13-14. c-MycKO B lymphocytes show the most dramatic phenotype when comparing to MaxKO and DKO cells. The absence of c-Myc in B lymphocytes seems to uncover the opposite role of Max by binding to E-boxes likely in association with proteins of the MXD family or MGA [3,4].

I. Page 12 - "c-Myc has a marginal role in primary B cells". This is an incorrect statement - right?

The original sentence on the manuscript is "*c-Myc ALONE has a marginal role in primary B cells.".*

Based on our new data regarding Myc expression in Max KO B cells, we have modified the text on page 14, to avoid any misinterpretation by "Our data suggest that c-Myc/Max heterodimers are the main effectors of Myc functions in primary B lymphocytes.".

J. Typos in text and Fig Legends - additional proofreading is needed.

We have fully revised the text and Fig legends.

Response to referee # 2

Major points

1. *The mixed genetic background (see Materials and Methods) materially detracts from this study as differences in the expression of genes other than Myc or Max may have impacted upon the phenotypes. This possibility should be acknowledged as a caveat to conclusions drawn about differences in phenotype and gene expression.*

We acknowledge that the mixed genetic background can affect the phenotype of mice and expression of genes other than Myc and Max. We have incorporated this possibility in the discussion on page 12 and 13.

2. *For each Cre transgene, efficiency of Max (and Myc) deletion (GFP-positivity) should be indicated and also assessed by PCR and western blot analysis.*

***mb1-cre* mice:** We have used these mice to analyze c-Myc/Max function only in the BM. c-Myc expression occurs during the proliferative expansion from pro-B to pre-B cell stage ($B220^{+}IgM^{-}$) in the BM[9]. It is at this stage where *mb1-cre*-mediated deletion occurs [10]. Western blot and qPCR analyses will detect protein and mRNA of c-Myc and Max since the deletion is an ongoing process at this stage. Therefore, we think that western blot analyses at these cell stages are unnecessary. Immature ($B220^{lo}IgM^{+}$) and recirculating mature B cells ($B220^{+}IgM^{+}$) (fig 1B and E) express extremely low levels, if any, of c-Myc in the BM [9]. In addition, *MycKO-mb1, MaxKO-mb1 and DKO-mb1* mice have very low numbers of these populations (Fig 1G) making technically challenging to perform Western blots on these cells after cell sorting by flow cytometry (50% recovery). Alternatively, to address this issue we have performed genomic PCR and qPCR analyses to monitor the deletion and expression of *c-myc* and *max*, respectively, on cell sorted immature and mature cells in the BM. These experiments showed very efficient deletion of *c-myc* and *max* in *mb1-cre* bearing mice. These data are shown in Fig EV1A and EV1C.

***cd19-cre* mice**: In supplementary Fig. 1A, currently Fig EV1B, we already showed genomic PCR analyses of *max* and *c-myc* deletion of sorted $B220^{+}GFP^{+}$ mature B cells from *HET-cd19, MycKO-cd19, MaxKO-cd19 and DKO-cd19* mice. As requested, we have performed *max* and *c-myc* expression analyses on these cells by qPCR. These new data are shown in Fig 5C. We have also carried out Western blot analyses of c-Myc and Max on the different genotypes carrying the *cd19-cre* modification. These data are shown in Fig EV1D.

3. *The text should explain the reason for switching from Mb1-Cre to CD19 Cre mice subsequent to the analyses shown in Figure 1.*

We have extensive experience with both *mb1-cre* [11] and *cd19-cre* mice [12]. The reason to use *CD19-cre* mice is a technical/practical one. *CD19-cre* mice provide a higher absolute number of deleted B cells in the spleen than *mb1-cre* to perform the experiments. *CD19-cre* mice are inefficient in Cre-mediated recombination in the bone marrow [13]. Since deletion of *c-myc* or *max* in bone marrow has a dramatic

effect in the generation of B lymphocytes, we observe that a large number of non-deleted B cells in *CD19-cre* mice "escape" and migrate to the spleen where they undergo Cre deletion of *max* or *c-myc*. At these stages Max or Myc are not necessary for maintenance of mature B cells and thus provide a higher number of B lymphocytes. To clarify this point, we modified the text on page 7 as follows: " To test the functional interplay between Max and c-Myc in this process we used *Cd19-cre* mice. These mice provide higher absolute numbers of deleted mature B lymphocytes in the spleen for analysis [12] than *mb1-cre* mice [11]."

4. *Figure 1*

Data for MycKO should have been included to compare with that of the MaxKO and DKO mice. It is insufficient to rely on historical data.

We have repeated the experiments shown in Fig 1 including *MycKO* mice as requested. We have double checked our data and a new statistical analysis has been performed to include *MycKO* mice (Figure B-J). The text and numbers have been modified accordingly on page 6.

- -Indicate the age and number of independent mice of each genotype that were immunophenotyped. Were they sex matched?

All mice used in these studies were 8-10 weeks old and the number of mice is indicated in the figure legends. The mice were not sex matched (very difficult with up to 4 genetic modifications). The missing info has been included in the figure legends.

- - 1F Define what is meant by total B cells - B220+ or B220+Ig+ ? There appears to be either a calculation error or the labelling is too poor in Fig1F, G, H to interpret. Fig 1F shows a <2x decrease in BM and spleen B cells in the DKO, yet the more precise data in panels G and H show much stronger deceases in DKO (>10x). Moreover, if one adds up the numbers of the Bcell fractions in panel 1G or 1H they are much lower than the total B cell numbers in panel F.

The total B cell number was defined as $B220^{+}GFP^{+}$ cells in the spleen or BM. We have included this info in the figure legend. The reviewer is absolutely right. There is a calculation error. Thus, we have re-analyzed all our data and included *MycKO-mb1* mice. We also have split panel 1F to allow better comparison.

5. *Figure 2*

- Indicate the statistical significance for MaxKO-CD19 and DKO-CD19 in comparison to both HET-CD19 and MycKO-CD19.

The information was originally provided at the end of Figure legend 2. However, the way was formulated might be misleading. To avoid this, we have modified the figure legend to clarify this point. "statistical analysis: two-tailed unpaired Student *t*-test (Prism 6.0; GraphPad). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. "

6. *Conclusion on page 7 end of 1st para should presumably be 'However, in the absence of Max, Myc or both, B cells in CD19-Cre mice can still generate ASC and perform CSR.'*

In our previous report [12], we observed a dramatic impairment in CSR and generation of ASC in c-Myc-deficient B cells. We always find a more dramatic phenotype in c-Myc deficient B cells than in DKO or MaxKO B cells. To stress that difference we wrote "*However, in the absence of Max or c-Myc/Max B cells can still generate ASC and perform CSR "* better than *'However, in the absence of Max, Myc or both, B cells in CD19Cre mice can still generate ASC and perform CSR.'* as suggested by the referee#2.

7. *Figure 4. The conclusion that loss of Myc/Max "drives cells to a hyperactive state" seems dubious. The KO cells do not proliferate well, are smaller (decrease FSC in fig.3D) and processes such as ribosomal pathway, translation, oxidative phosphorylation and metabolism are down-regulated. The authors do show that some signaling pathways might be up in KOs but this might simply be due to the lack of differentiation compared to the Het control.*

This point was raised also by referee #1 and #4. See response to referee#1 (point 3E).

8. *Figure 3.*

-legend states that data in A are representative of at least 3 independent experiments. How many mice were used in each arm of each experiment? Gating of cell cycle analysis in 3E is strange. Not sure how they define the S phase as the dashed line seems too high. The gating of the same type of data in Figure 1I is much better and very different from 3E.

The dashed line in Figure 3E is not intended to define the S phase. It is an arbitrary line to show the difference in EdUMean fluorescence intensity. The percentages of the different stages of the cell cycle were calculated using FlowJo software. We have added the different cell cycle phases and clarified this point in the Figure legend 3E. Number of mice has been added to the figure legend. OK

Minor points

1. *Vallespinos et al is missing from the reference list.*

We have double-checked the submitted manuscript. According to our file, the reference is on page 25 included in the reference list.

2. *Flow cytometry is two words rather than one.*

We have changed flowcytometry by flow cytometry.

3. *The authors should comment on whether N-Myc or L-myc are expressed in this system and possibly complicate the interpretation. They have this in their RNAseq data*

We checked the RNA-Sequencing data for *n-* and *l-myc* expression. We did not observe significant differences between all the mutants and the heterozygous controls. Thus, we conclude that there is not functional compensation between the different Myc family members. This information has been included on page 13.

Response to referee # 3

1. *However, I ask the authors to conduct the following experiments to address my concern. I felt weird that the authors did not show data obtained form cMyc-KO-mb1 in this manuscript, although they showed such data previously. I consider that the authors should simply show phenotypic differences between c-MycKO-mb1 and MaxKO-mb1 by providing both in Figure 1 of this manuscript.*
We have repeated the experiments in Fig. 1 to include *MycKO-mb1* mice data for comparison. This was also requested by Referee#2 (point 4).

2. *In addition, I strongly recommend to change the title to the one that represent their findings clearly.*
We agree with referee #3 that the title might fall short on describing the relevant results. However, due to the number of biological processes analyzed it has been really difficult to come up with a good sentence that summarizes our findings. We think our current title exposes up front the biological question to the reader and thus facilitates the understanding of the manuscript. However, we propose the following titles:
"B lymphocytes do not require c-Myc and Max to initiate essential biological functions"
"c-Myc and Max are required to fine-tune the transcriptional response after activation in B lymphocytes"

3. *Ideally, it is better to explore whether MAX requires additional factor such as Mad to exert detrimental phenotype of cMyc-KO B lymphocytes.*
We agree that exploring whether MAX requires additional factors of the MXD family (Mad) or even MGA would likely provide new insights on the MYC/MAX-dependent mechanisms operating in B lymphocytes. However, we feel that our data is the first step to study these interactions. In addition, the numerous members of the MXD family would raise the possibility of functional compensation between the different factors.

Response to referee # 4

Major concerns:

1. *Figure 1-4: Lack of Myc/Max deletion efficiency data on protein and mRNA level.*

This concern was also raised by referee #2. We have performed qPCR and Western blots for Myc and Max. See response to referee #2 point 2.

2. *It is thus important to show what is the nature of Max protein expression upon Cre mediated deletion (a truncated, unstable, Myc binding protein?)*.

HLHZip domain is essential for dimerization with Myc proteins. We have included this comment on page 4 *"Cre recombinase deletes exon 4 and 5 containing HLHZip domain and 3´UTR (Fig. 1A)"*. In addition, in the M&M section "Max protein contains 5 exons encoding 160 aminoacids. Cre recombinase deletes exon 4 and 5 (aa 57-160) containing HLHZip domain and 3´UTR."

3. *Figure 1. B-J: Lack of MycKO-mb1 controls.*

Figure 1. I-J: Lack of Myc/MaxDKO-mb1.

Referee #1, point 3A, #2, point 4, and #3, point 1 raised similar concerns. We have repeated all the experiments in Figure 1 to include *MycKO-mb1* and *DKO-mb1* mice. See response to Referee #1 (point 3A).

4. *Figure 2. A-H: Lack of deletion efficiency data in d3 B cell cultures and sorted ASC and IgG1+ B cells. Given the very low efficiency of plasma cell differentiation and CSR in the mutants, it is absolutely critical to show that the observed cells are indeed Myc/Max knock-outs.*

Indeed, we looked at deletion of *c-myc* and /or *max* in those cells. The data are shown in figure EV1B (previously supplementary Fig1). We clearly failed to indicate the origin of those cells in the figure legend and apologize for that. Accordingly, we modified the figure legend as follows: " **(a)** Genomic PCR analysis of *wt*, *deleted* and *flox* alleles of *HET-cd19, MycKO-cd19, MaxKO-cd19 and DKO-cd19* genotypes from sorted day 3 $B220^{+}GFP^{+}$ spleen cells used in Fig. 2. In addition, we have performed qPCR and Westerns blots on *mb1*- and *cd19-cre* bearing mice. Data are shown in Fig EV1.

5. *Figure 2. I-J: Methods state that GCs were scored 30 days post immunization. That is an unreasonable time for primary B cell responses. Standard protocol is 10-14 days.*

We have repeated the immunization experiments in Fig. 2 scoring the GC at 13 days. In addition, to provide a more complete description, we have included *MycKO-cd19* and *DKO-cd19* mice for these analyses. The new data are shown in Fig. 2I and text has been modified accordingly on page 8.

6. *Figure 3. C-D: Bright field images of activated B cell cultures as an indicator of B cell size have very limited if any value. It is difficult to understand what the authors want to conclude from an FSC histogram plot, given the fact that the FSC hi blasting B cells (present in the control around the value of 400K) are completely absent in all KO plots.*

Our aim was to show whether the previously reported role of c-Myc in cell size in B lymphocytes [14] was mediated by Max or not. We have added a line for better comparison among the FSC histograms. We observed some modest differences between MycKO and MaxKO and DKO cells despite not having large blasts. We agree that bright field images have limited value but together with FSC histograms show that Max KO and DKO cells have some capacity to restore cell size. OK

7. *Figure 4. E, G: From RNA seq analysis of B cells activated with LPS+IL4 for three days in vitro the authors conclude that B cells lacking Myc and/or Max (whose absence is once again not shown) are in a "hyperactive state". This conclusion based solely on mRNA expression data, seems inappropriate, particularly in view of the comparable levels of the CD69 activation marker before and after activation in Figure 2G.*

A similar point was raised by referee #1 (point 3E) and #2 (point 7). Please see the response to referee #1 (point 3E).

8. *Supplementary Figure 4. A. Authors claim: "as an internal control for RNA-Seq, we observed a similar gene expression profile of previously reported genes (Fernandez et al. 2013", Fig 5). However, of 11 highlighted genes, expression of 5 does not correlate with the data of Fernandez et al.*

We highlighted 12 genes. We have re-evaluated the data shown in Fernandez et al. [12]. We observed that gene expression of 9 out 12 are in the "bulk part" and agreement with the results of this report. Among the three remaining genes, *pax-5* and *pou2f2* did not correlate with the data shown here and thus are no longer highlighted. In the case of *eif2ak*, we confused this gene with *eif2ak2*, and we apologize for this. We eliminated the text "Genes highlighted in red were previously tested by qPCR22…" from Figure legend EV4A. The different activation stimulus, anti-CD40 plus IL-4 [12] vs LPS plus IL-4 (this report) between both experiments might account for differences in gene expression profiles. On this regard and requested by Referee #1 (point 3G), we independently confirmed by qPCR some of the up or downregulated genes observed in the RNA-Seq data (Fig 5C).

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2nd Editorial Decision 16th Jul 18

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below).

As you will see, all referees now support the publication of your manuscript in EMBO reports. However, referees #1, #2 and #4 have several further suggestions to improve the manuscript, and a few concerns we ask you to address all in a final revised manuscript. Most importantly, the overall structure and flow of the manuscript needs to be improved (as indicated by referees #1 and #4), and the final text needs to be proofread by a native speaker.

Further, I have these editorial requests we ask you also to address:

- The abstract is currently too long. Please provide an abstract with not more than 175 words. Please also provide the abstract written in present tense.

- Please add scale bars to the images in Figure 2I and EV3A.

- Please display and cite the figures in a sequential manner throughout the manuscript. E.g., Fig. 5C is cited before Fig. 1 in the current version of the manuscript.

- The Western blot images in panel 3I sometimes differ very strongly in terms of contrast and brightness (background). Could you provide these Western panels as unmodified as possible and with similar background intensities? Further, the Western blot panels in Figure EV1A/B are of rather low quality and resolution. Could you provide higher resolution versions of these?

- Please add a paragraph describing the statistical analyses used in the manuscript to the M&M section.

- Please add the Gene Omnibus accession number for the RNA-seq. data to the respective section of the M&M section.

- Please add page numbers to the Appendix and the TOC. Please also use the correct nomenclature for the Appendix files. It should be Appendix Figure S1 and Appendix Table S1. Please update the respective callouts in the manuscript text.

- Thanks a lot for providing source data for Figures 3 and EV1D. Could also source data for panels EV1A/B be provided? Please combine these with those of EV1D in one file, and replace the old source data file for EV1 upon re-submission.

REFEREE COMMENTS

Referee #1:

I believe that the authors have adequately addressed my concerns. That being said, there are parts of the manuscript where the writing is not of high quality (the data, ideas and conclusions could be better and more fully expressed) and could benefit from being proofread by colleagues who speak and write fluently in English.

Referee #2:

The authors have addressed most of my comments satisfactorily. However, the Materials and Methods should clearly indicate the genetic background of each mouse strain used so the mixed genetic background is abundantly clear. Furthermore, a stronger statement should be made in the discussion to indicate that this mixed background could contribute to the phenotypic differences. In regard to the sentence replacing the term 'hyperactive state', it should be modified to read 'These

data suggest that the lack of c-Myc, Max or both in B cells results in up regulation of expression of genes involved in different signalling pathways.'

Referee #3:

I consider that the authors conducted enough experiments to address my concern.

I agree that what I asked about MXD family is beyond the scope of present study.

It is OK for the present title.

I don't have any further comments on this manuscript and I am willing to recommend publication of this manuscript in EMBO Reports.

Referee #4:

In this report the authors generate a conditional Max knockout mouse and examine the contribution of Max to Myc function in B cell development in vivo and B cell differentiation and class switching in vitro in compound mutants of floxed Myc, Max and Myc/Max alleles with B cell specific Cre strains (Mb1, CD19) in combination with R26-YFPflSTOP reporter.

The authors provide evidence that B cells in absence of Myc/Max heterodimers are partially able to sustain proliferation and differentiation into naïve resting splenic B cells in contrast to Myc only KO B cells. However, Myc/Max are equally essential for the generation of germinal centers upon T-dependent immunization.

The authors have added critical experiments providing evidence that the mature B cell populations present in Max and Myc/Max KO mice, although strongly decreased in numbers, are indeed mutants and not rare escapees of Cre-mediated deletion. However, these experiments are not properly discussed.

Upon TLR4 stimulation, B cells might require the Myc/Max heterodimers to limit the extent of signalling cascade activation. However, this point remains weakly supported by experimental evidence.

Myc seems to exert an important regulatory role on Max mediated gene expression, which might be a complicated story, given the fact that Myc seems to require the presence of Max for its stability.

Although the manuscript has been improved and addresses an interesting topic, it still lacks the quality required for publication in EMBO Reports.

Critical points:
Abstract should be carefully rewritten. Text and Figure legends should be improved.

Transcriptome analysis.
A well-defined scientific question, clear conclusions and proper discussion are missing / unsatisfactory.

The transcriptome analysis lacks data for time 0 of both control and mutant B cells.

Comparison of the transcriptome profiles of Myc KO, Max KO, Myc/Max KO with published results from primary B cells and CD8 T cells does not give any clear message.

It is unclear why the authors decided to concentrate on the genes whose expression overlaps between the three knockouts.

Results confirming the role of Myc/Max in well-known Myc-dependent pathways such as ribosome biology, regulation of transcription and translation, and metabolism (e.g. Sabo et al., Nature 2014)

are not discussed properly.

The authors have to demonstrate that the class switched cells (1-3%) and plasmablasts (0.3-1%) obtained at d3 are indeed mutants by PCR analysis on sorted subsets (plasmablasts and IgG1+ cells). Antibody secreting cells or plasma cells are by definition cells that secrete antibodies. If the authors do not provide evidence for antibody secretion (by ELISA or ELISPOT), but show only the surface markers, these cells should be termed plasmablasts.

Figure legends: What do the columns represent, mean or median values? Do error bars represent standard deviation or standard error of mean?

Figure 1.
A. missing PCR strategy
B-H. poorly described in figure legend and in the main text.
F. Which bone is the origin of the BM?
Should the y axis read "Cells (x10^7)" instead of "Cells (x10^6)" ?
G. Which bone is the origin of the BM?
H. How are immature splenic B cells defined? AA4.1+? IgD-?
I. S phase MFI measurements of EdU reported in text do not match with dot plots.

EV2. GFP histogram at d4 is missing, deletion efficiency on sorted IgM+ B cells at d4 is missing, Myc ko and DKO controls are missing

Figure 2.
B., D., F., H., J. The format to show statistical significance should be kept as in Fig 1F.
G. Gate should be corrected in DKO-cd19.

EV3D. Are the absolute numbers GFP+ or total GC cells?

Figure 3.
A. Were the cells sorted as B220+IgM+GFP+ (as stated in text) or B220+ GFP+ (as stated in figure legend)?
E. Authors should comment on the surprising differences of the S phase entry and EdU MFI observed between Fig 3E (in vitro) and Fig 1I (in vivo pro/pre BM B cells).
Are the differences in cell cycle distribution between mutants statistically significant?
Quantification of EdU MFI is missing.
H. Statistics missing.
I. Loading controls should be mentioned in figure legend.

Figure 4.
A. Define DEGs as +/- 1.5 FC and $p<0.01$ and show separate Venn diagram for UP- and DOWN-regulated genes.
C. What do the columns "Gender Cond" represent?
D. What does the color code scale represent?

EV4A: DNAJB9 expression does not correlate with the referenced paper

Figure 5.
A. What is the FDR cutoff for KEGG pathway analysis?
B. What does the heatmap color code scale represent?
C. Expression of selected genes (mrpl12 and rpl39) measured by qPCR does not confirm stronger downregulation in MycKO-cd19 than MaxKO-cd19 or DKO-cd19 B lymphocytes.

EV4C. For this analysis a gene set of 850 Myc-only regulated genes (Fig 4B) should be used.

Editorial requests

1. *The abstract is currently too long. Please provide an abstract with not more than 175 words. Please also provide the abstract written in present tense*. Abstract has been shortened to 175 words and the whole manuscript professionally edited.

2. *Please add scale bars to the images in Figure 2I and EV3A*. Scale bars have been added to images in Fig 2I, EV3A and figure legends.

3. *Please display and cite the figures in a sequential manner throughout the manuscript. E.g., Fig. 5C is cited before Fig. 1 in the current version of the manuscript*. The sequential order of the figures has been fixed.

4. *The Western blot images in panel 3I sometimes differ very strongly in terms of contrast and brightness (background). Could you provide these Western panels as unmodified as possible and with similar background intensities? Further, the Western blot panels in Figure EV1A/B are of rather low quality and resolution. Could you provide higher resolution versions of these?*. Western blot images in panel 3I has been modified for contrast and brightness. Western blots in Fig EV1A/B are provided with a better resolution.

5. *Please add a paragraph describing the statistical analyses used in the manuscript to the M&M section.* The statistical analyses have been added to M&M.

6. *Please add the Gene Omnibus accession number for the RNA-seq. data to the respective section of the M&M section*. The accession number has been added to the corresponding section in M& M.

7. *Please add page numbers to the Appendix and the TOC. Please also use the correct nomenclature for the Appendix files. It should be Appendix Figure S1 and Appendix Table S1. Please update the respective callouts in the manuscript text*. These issues have been fixed.

8. *Thanks a lot for providing source data for Figures 3 and EV1D. Could also source data for panels EV1A/B be provided? Please combine these with those of EV1D in one file, and replace the old source data file for EV1 upon re-submission.* The source data Panels EV1A/B are provided and merged with EV1D.

Response to referees

We thank all the referees for their suggestions and comments.

Referee#1

1. *There are parts of the manuscript where the writing is not of high quality (the data, ideas and conclusions could be better and more fully expressed) and could benefit from being proofread by colleagues who speak and write fluently in English*. The manuscript has been now professionally edited by a native speaker.

Referee#2

1. *Materials and Methods should clearly indicate the genetic background of each mouse strain used so the mixed genetic background is abundantly clear. Furthermore, a stronger statement should be made in the discussion to indicate that this mixed background could contribute to the phenotypic differences.* We have included the genetic background of all the mouse models use in this study in M&M. In addition, we have modified the sentence in the discussion to provide a stronger statement on page 14. Specifically, "*It should be noted that the mixed background of*

these models can play an important role in the observed differences in phenotype and gene expression."

2. *In regard to the sentence replacing the term 'hyperactive state', it should be modified to read 'These data suggest that the lack of c-Myc, Max or both in B cells results in up regulation of expression of genes involved in different signaling pathways*." We have modified the sentence as requested on page 12.

Referee#3

No points to be addressed.

Referee#4

1. *Abstract should be carefully rewritten. Text and Figure legends should be improved*. The manuscript has been now professionally edited by a native speaker.

2. *Transcriptome analysis: A well-defined scientific question, clear conclusions and proper discussion are missing / unsatisfactory***.** We have re-written the specific sections and professionally edited the manuscript.

3. *The transcriptome analysis lacks data for time 0 of both control and mutant B cells*. We think that time 0 would not provide more useful information since the cells are in a "resting", non-activated state. No activation markers are seen at time 0 (Fig 2G, control cells, day 0) and thus the most striking phenotype of our study would not be observed. In addition, *c-myc* is not expressed in resting B lymphocytes.

4. *Comparison of the transcriptome profiles of Myc KO, Max KO, Myc/Max KO with published results from primary B cells and CD8 T cells does not give any clear message*. We have modified the text on page 13 to clarify the message. Specifically, we have added the following sentence: "*We observed that metabolic and ribosome pathways were downregulated whereas signaling pathways such as NF-κB and MAPK were clearly upregulated in T lymphocytes lacking c-Myc (Fig EV5B)*"

5. *It is unclear why the authors decided to concentrate on the genes whose expression overlaps between the three knockouts*. In general terms, it is widely assumed that c-Myc requires Max. We focused our analyses on genes up- or-downregulated in the three experimental conditions because this implies that the gene expression changes required the presence of the heterodimer c-Myc/Max as a starting point of our study. In addition, under these conditions we observed the majority of the gene expression changes.

6. *Results confirming the role of Myc/Max in well-known Myc-dependent pathways such as ribosome biology, regulation of transcription and translation, and metabolism (e.g. Sabo et al., Nature 2014) are not discussed properly*. We have discussed in more detail all our RNA-seq data.

7. *Figure legends: What do the columns represent, mean or median values? Do error bars represent standard deviation or standard error of mean?*. The columns represent mean values, and the error bars standard deviations. This information has been added in the figure legends.

8. *The authors have to demonstrate that the class switched cells (1-3%) and plasmablasts (0.3-1%) obtained at d3 are indeed mutants by PCR analysis on sorted subsets (plasmablasts and IgG1+ cells)*. In figure EV1B, we showed that GFP^+ B lymphocytes of all genotypes had *c-myc*, *max*, or both alleles deleted at day 0. The flowcytometry analyses showed >99% of the cells were GFP^+

at day 0. Moreover, the analyses at day 3 are performed on GFP^+ cells. Thus, we think it is unnecessary to perform the experiment to show the deletion again.

9. *Antibody secreting cells or plasma cells are by definition cells that secrete antibodies. If the authors do not provide evidence for antibody secretion (by ELISA or ELISPOT), but show only the surface markers, these cells should be termed plasmablasts.* We have modified the text to replace Antibody Secreting Cells (ASC) by plasmablasts.

10. *Figure 1*

A. missing PCR strategy. We have added the PCR primers in figure 1A and a detailed strategy in Fig EV1A.

B-H. poorly described in figure legend and in the main text. We have modified the figure legend and text.

F. Which bone is the origin of the BM?. The information has been incorporated in the figure legend.

Should the y axis read "Cells (x10^7)" instead of "Cells (x10^6)" ?. The correct Y axis is "Cells (x10^6)" because we show GFP^+ cells.

G. Which bone is the origin of the BM?. We have added the info in the figure legend.

H. How are immature splenic B cells defined? AA4.1+? IgD-?. It was already shown in M&M main text. We have added this info in the figure legend.

I. S phase MFI measurements of EdU reported in text do not match with dot plots. Referee # 4 is right. MFI values of S phase EdU are wrong in the main text, it should be: "*EdU incorporation of sorted GFP^+ B lymphocytes revealed a reduction in cells in S phase in pro- and pre-B stages and a decreased intensity of the EdU fluorescence signal in MaxKO, MycKO and DKO versus control cells (S phase mean fluorescence intensity (MFI): 19886 HET vs 5172 MaxKO, 4017 MycKO, 4416 DKO) (Fig 1I)"*. We corrected the main text. The previous MFI values were from the total EdU Y axis, not only the S phase, as the main text said.

11. *EV2. GFP histogram at d4 is missing, deletion efficiency on sorted IgM+ B cells at d4 is missing, Myc ko and DKO controls are missing*". We have modified the figure and provided the GFP histogram for day 4. At day 0 cell sorting was carried out with purity >99% of GFP^+ cells. As routinely observed, some of the B lymphocytes die in culture after 4 days and thus, loose their GFP signal. Consequently, we observe two peaks of GFP corresponding to alive (GFP^+) and dead cells (GFP^-) at day 4. In addition, the analysis of B cell differentiation is performed gating on GFP^+ B lymphocytes that we previously have shown have *max* alleles deleted in Fig EV1B. Therefore, we think that the deletion efficiency at day 4 is not necessary.

c-Myc KO B cells do not differentiate to $B220^+IgM^+$ lymphocytes as we already published (1) and referenced on the main text. Finally, we acknowledge that DKO could provide some additional information but we have been very careful not to overinterpret our data and restrict our discussion to MaxKO B cells.

12. *Figure 2*.

B., D., F., H., J. The format to show statistical significance should be kept as in Fig 1F. We have modified the format as suggested for panels 2B, D, and J. In panels F and H differences are not statistically significant and thus modifications are not necessary. Due to the nature of the data and space constraints, only panel J has not been modified.

G. Gate should be corrected in DKO-cd19. We have corrected it and apologize for the mistake. Numerical values were correct and have not been modified.

EV3D. Are the absolute numbers GFP+ or total GC cells?. The absolute numbers are total $GFP^+B220^+GL\text{-}7^+$. We have added this information in the figure legend.

13. *Figure 3*.

A. Were the cells sorted as B220+IgM+GFP+ (as stated in text) or B220+ GFP+ (as stated in figure legend). Cells were sorted as $B220^+GFP^+$. We corrected the mistake and apologize for it.

E. Authors should comment on the surprising differences of the S phase entry and EdU MFI observed between Fig 3E (in vitro) and Fig 1I (in vivo pro/pre BM B cells). We have discussed these differences on the main text on page 9-10.
Are the differences in cell cycle distribution between mutants statistically significant?. These differences are not statistically significant.
Quantification of EdU MFI is missing. We have added MFI values in the figure legend.
H. Statistics missing. Statistics have been incorporated in the figure and the statistical method used in the corresponding M&M section.
I. Loading controls should be mentioned in figure legend. We have included this information in the figure legend.

14. *Figure 4*.
A. Define DEGs as +/- 1.5 FC and p<0.01 and show separate Venn diagram for UP- and DOWN- regulated genes. DEG were defined as +/-1.5 FC and $p<0.01$. A separate Venn diagram is shown in the figure 4.
C. What do the columns "Gender Cond" represent?. Gender represent mouse gender. Pink are female and blue are male mice. This information has been added in the figure legend. Cond represents code colour for each genetic condition (HET, MaxKO, MycKO and DKO). This information is redundant and has been removed from the figure.
D. What does the color code scale represent?. Colour code: red represents upregulation, white, unchanged, and blue downregulation of expression. This information has been incorporated in the figure legend.

15. *EV4A: DNAJB9 expression does not correlate with the referenced paper*. We have corrected the mistake. We have unlabeled it.

16. *Figure 5*
A. What is the FDR cutoff for KEGG pathway analysis?. FDR <0.05 for up- or down-regulated genes common for all three conditions (≥1.5-fold and $p<0.01$). We have added this information in the figure legend.
B. What does the heatmap color code scale represent?. Colour code: red represents upregulation, white, unchanged, and blue downregulation of expression. This information has been incorporated in the figure legend.
C. Expression of selected genes (mrpl12 and rpl39) measured by qPCR does not confirm stronger downregulation in MycKO-cd19 than MaxKO-cd19 or DKO-cd19 B lymphocytes. We acknowledge that we did not observe stronger downregulation in MycKO-cd19 B cells as observed in the RNA-seq data. These differences could be due to the different techniques used qPCR vs sequencing or primer design.

17. *EV4C. For this analysis a gene set of 850 Myc-only regulated genes (Fig 4B) should be used.*
The main argument to analyze the data as shown in Fig EV4C was to assume that individual genes deregulated under the 3 conditions (MycKO, MaxKO and DKO), with a significative logFC, required the functionality of c-Myc and Max as a Myc/Max heterodimer. Those 850 "Myc-only genes" shown in Fig 4B are down or up-regulated genes with no significative expression alteration in MaxKO or DKO. Thus, these genes are not "genuinely" c-Myc only genes. Even if activation or repression of a gene by c-Myc did not require Max, we would expect that in DKO cells its expression would be altered. Thus, we think that the 45 down- and 49 up-regulated genes analyzed in Fig EV4C represent truly c-Myc-regulated genes, that do not require Max.

Reference

1. Vallespinos M, Fernandez D, Rodriguez L, Alvaro-Blanco J, Baena E, Ortiz M, Dukovska D, Martinez D, Rojas A, Campanero MR, *et al.* (2011) B Lymphocyte commitment program is driven by the proto-oncogene c-Myc. *J Immunol* **186:** 6726-6736

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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: IGNACIO MORENO DE ALBORAN
Journal Submitted to: EMBO REPORTS
Manuscript Number: EMBOR-2018-45770-T

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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?	The sample size was chosen based on guess based on previous experiments. For flow cytometry and in vitro experiments, we repeated the experiments no less than three times to obtain at least (mice, n=3) for each genotype.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size mice was estimated following the recomendations of the CSIC-Ethical comitte and approved by the Madrid regional goverment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No pre-established exclusion criteria were established. No animals were excluded from analyses
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.	No ramdomization protocol was applied.
For animal studies, include a statement about randomization even if no randomization was used.	No ramdomization protocol was applied.
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.	Yes. The experiments involving RNAseq were coded and blinded to the investigator.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes. The experiments involving RNAseq were coded and blinded to the investigator
5. For every figure, are statistical tests justified as appropriate?	YES.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES. Two-tailed unpaired Student t-test. All the statistical methods are indicated in the figure legends. For RNA-seq data, the specific statistical analyses performed are indicated in the corresponding section in M&M.
Is there an estimate of variation within each group of data?	YES. This info is provided in the figure legends.
Is the variance similar between the groups that are being statistically compared?	YES.

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).	YES. This information is provided in M&M section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	This info is provided in figure legends and M&M section. Mice were generated in our transgenic facility. The animal facility complies with all the regional, national and european law for animal husbandry. Especialized personnel takes care of all duties concerning the well being of the mice.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have all the permits required for all the procedures carried out during these experiments. Specifically, we have approval from the CSIC Ethical committe for animal experimentation and from the Madrid regional goverment.
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.	OK.

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 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	YES. RNA-sequence data will be deposited in gene expression Omnibus and aacession code provided at the end of M&M.
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).	Relevant and complete Western Blots are provided according to EMBO reports policy.
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.	NA