EMBO reports

An early Myc-dependent transcriptional program orchestrates cell growth during B-cell activation

Alessandra Tesi, Stefano de Pretis, Mattia Furlan, Marco Filipuzzi, Marco J. Morelli, Adrian Andronache, Mirko Doni, Alessandro Verrecchia, Mattia Pelizzola, Bruno Amati and Arianna Sabò

Review timeline:

Submission date:	24 February 2019
Editorial Decision:	8 April 2019
Revision received:	13 May 2019
Editorial Decision:	13 June 2019
Revision received:	18 June 2019
Accepted:	27 June 2019

Editor: Deniz Senyilmaz-Tiebe

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 8 April 2019

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

I apologize for the delay in getting back to you. It took longer than anticipated to receive the full set of referee reports.

As you can see, all referees express interest in the presented function of Myc in regulating the transcriptional remodeling upon B-cell activation. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here.

Given these constructive comments, I would like to invite you to revise your manuscript with the understanding that the referee must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If

the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

REFEREE REPORTS

Referee #1:

In the manuscript the authors undertake a very detailed analysis with a particular emphasis on the early response during LPS stimulation of mature B cells. The authors find that the early response to LPS stimulation involves only a few hundred genes, rather than amplification of the entire transcriptomic signature (amplifier model), as argued by other investigators.

The data and analysis are very well organized and presented. The manuscript does not provide any particularly novel findings regarding the function of Myc, as previous studies, well referenced by the authors, have addressed both the Myc amplifier model and the other findings presented in this study. Nonetheless the data do strongly support the argument against the controversial notion that Myc acts as a global amplifier of transcription in B cell activation. Furthermore, the detailed time-course following LPS stimulation represents a unique resource.

Specific Comments

Figure 1. Figure 1E apparently largely depends on an analysis of RNA synthesis rates that are modeled, but not measured. However, validation of this method appears to be lacking (e.g. comparison of the data derived from this method to a pulse measurement, such as 4sU labeling). Without this, the pulse data cannot be considered rigorous.

Figure2. The authors point out that Myc binds only to pre-existing promoters/enhances that are essentially marked as active. They call this 'open chromatin'. Open chromatin is a somewhat vague term that can involve many epigenetic marks, and there are certainly studies that indicate Myc has some role in this process (Kieffer-Kwon, et. al., 2017). This current study shows no evidence of global chromatin structure change at these early time points. Perhaps a more specific term such as 'active promoters' should be used.

In Figure 2H, the data are clear that canonical E-boxes are enriched in cells expressing the lowest levels of Myc (0h), as expected for high affinity sites. Yet this is only a relatively minor fraction (~25%) of the signature. In fact, it appears that approximately 40% of the peaks do not contain even degenerate E-boxes yet would be classified as high affinity sites (since they're bound at lowest Myc). The authors should comment on these promoters, since they really don't behave according to the model the authors suggest (text, top of page 6).

Figure 4. When assessing recruitment of Pol2 vs elongation, the authors make the important point that recruitment of Pol2 to promoters is the factor that has the highest correlation with differentially expressed genes, even more so than the effects on pause-release. Also, subsequent Pol2 elongation steps should also be dependent on recruitment. The authors use an algorithm to model recruitment and subsequent pause-release and elongation steps. The authors should at least mention that the elongation data might be better addressed using more powerful techniques like ProSeq or 4sU-FP-seq (Liang, and Shilatifard., Cell, 18 October 2018) to map Pol2 with high resolution and pulse methodology.

An interesting point here is that Myc is generally lost at genes that are down-regulated by LPS. This is termed a 'passive process', but Myc levels are going up (why aren't these high affinity sites in LPS?). Therefore, the model in which Myc occupies sites based on low/high affinity and Myc levels isn't well explained by this data. This warrants further discussion or investigation as to what is the mechanism involved in this effect (e.g. other histone modifications, chromatin structure, etc.).

Minor points

The representative western blot in EV1 isn't very high quality because the Myc bands are so broad. The data would be improved with a higher quality blot.

Referee #2:

In this report, Tesi, de Pretis et al. address a basic question - what is the role of the transcription factor Myc in the early hours of B cell activation - and they provide yet another piece of evidence that, instead of being a non-selective transcriptional amplifier, a regulator of cell cycle or DNA replication, Myc recruits RNA polymerase to a well-defined set of genes responsible for RNA metabolism and energy production which prepare the cells for future growth and proliferation.

Their experimental system is simple, Tat-cre mediated deletion of floxed Myc alleles in primary B cells followed by LPS stimulation which they characterize in sophisticated detail by RNAseq and ChIPseq methods and thorough bioinformatic analysis. I particularly appreciate the great emphasis they put to assess the deletion efficiency and identify the extent of Cre-mediated deletion escapees.

The report by Tesi, de Pretis et al. is of interest to a broad scientific community, particularly scientists interested in Myc-mediated gene regulation, but also B cell activation.

This is a solid scientific report which has logical flow. It is well written, although some parts need further/better explanation.

I have 2 comments and minor points I would ask the authors to address:

Comments:
1)
The authors observe a correlation between the gain of Myc binding at the promoters (Myc share) and the increase of RNA synthesis from these promoters. This correlation is the strongest for Myc-dependent genes, although still present at Myc-independent loci (Figure 3 a,b). The discussion of the "at first sight paradoxical" findings and "the notion that promoter activity and Myc binding are mutually dependent" is confusional and appears contradictory to the finding of Myc-dependent and -independent set of genes. The authors should improve the discussion and better explain what they mean.

2)
To "increase the resolution of the analysis" the authors introduce a clustering approach in Fig 4. From this point on, the flow of the paper becomes very complicated for 3 reasons:

• The aims of the clustering approach are poorly explained
• Clusters (numbers and properties) are poorly described
• The selection criteria of only certain clusters for further analysis are not clearly explained.
The authors should restructure and better explain this section.

Minor points:

Figure 1.
B) Provide your own measurement of cell size at 0, 12, 24 h in Myc wt and ko. Is "Nuclear Area" the nuclear size? Dots represent individual cells?

C) The numbers which should help the reader identify gene subsets are hard to read. Define the "grey zone" in the text. The different regulatory groups should be clearly defined in the text.

Figure EV1
Is the cell survival of Myc wt and ko cells comparable at different time points upon TAT-cre transduction?
D) To better understand the binding dynamics of Myc, show ChIP qPCR for 2, 4 and 8 hours in Myc

ko and wt cells. Does Ncl contain E boxes? Can you confirm higher binding of Myc on E-box promoters as compared to promoters that do not contain E-boxes by ChIP qPCR?

Figure 2
B) The CpG mentioned in the figure legend is not found in the figure.
G) This figure needs better explanation in text and figure legend. What is the color code and rows?

Figure 3
B) This graph is not easily comprehensible without a brief explanation in the text. Moreover, the axis, sensitivity and specificity shall be explained in the figure legend. What is the grey line? Dependent and independent stands for Myc-dependent and -independent?

Figure EV 3 C, D); Figure 4 F-H); Figure EV 4, 5 missing statistics.

Figure EV6 A short explanation is needed in text.

Minor suggested text edits:
p.6
...in either B-cells (Guccione, Martinato et al., 2006, Lin et al., 2012, Nie et al., 2012, Sabò et al., 2014) or other cell types ...

p.7
... Myc drove rapid and selective activation of high-affinity promoters, most frequently associated with the presence of the E-box binding motif.

p.9
...we have identified approximately 650 genes that were induced in a Myc-dependent manner within 4-8h following B cell stimulation by LPS, and an additional group of ca. 1100 regulated by Myc with modest quantitative...

... Interestingly, the Myc-dependent program identified here in activated B-cells was constitutively deregulated during lymphomagenesis in Eμ-myc transgenic mice (Sabò et al., 2014)...

Referee #3:

The study by Tesi et al aims to delineate early Myc-dependent gene expression profiles during B-cell activation. To do so, they compared c-myc+/+ and c-mycf/f primary B cells stimulated by LPS following exposure to tat-Cre recombinase. A major conclusion is that, during LPS-driven B-cell activation, MYC drives rapid and selective activation of several hundred high affinity promoters, through further RNAPII recruitment, and that the genes affected encode proteins involved in RNA biology, energy production and anabolic pathways. By comparing profiles obtained after LPS stimulation with those they have previously reported for pre-malignant and malignant B lymphoid cells from Eμ-myc mice (Sabo et al Nature 2014), they conclude that the same genes are activated in B lineage cells driven by oncogenic high levels of MYC. They conclude that MYC has no direct impact on global transcriptional activity, contrary to the amplifier model of MYC activity.

The paper deals with an important and still-controversial biological issue: the mechanism of action of MYC. In general it is well-written, succinctly presented and provides original data that will be of interest to the many researchers studying MYC. I recommend publication, contingent on consideration of the following comments and appropriate minor revision.

1. Abstract line 5-6. Description of protocol is misleading. Change to, for example, 'We addressed this in LPS-stimulated mouse B-cells, by comparing WT versus c-myc-deleted cells'.

2. To my mind, insufficient attention is paid in the paper to characterising the cell populations used for LPS treatment and RNA profiling. FACS profiles should be provided showing the viability, cell size and immunophenotype before and after treatment with tat-Cre. I would also like to see evidence of c-myc deletion by Southern blot or PCR. Was the Cre protein used the His-Tat-NLS-Cre described by Peitz et al?

3. How many independent batches of cells were analysed?

4. p4 para 2
- Line 8.The case is overstated - only Ncl and Pus 7 expression are convincingly Myc-dependent. Modify sentence accordingly.
- indicate in text that the B cells are isolated from spleen

5. Figure 2
- 2a: description in text and legend is inadequate to enable the reader to understand this diagram.
-2b: Why were chromosome 1 Myc-binding promoters selected? Were similar results obtained for sites on other chromosomes? In any case, I would be cautious re the comparison of the LPS data obtained here for splenic B cells isolated by magnetic bead depletion of non-B cells versus those reported in Sabo et al (2014) for control WT B-cells (C; enriched by binding to B220+ beads), premalignant splenic cells from age-matched (6-8wk) Eμ-myc mice (P; enriched by binding to B220+ beads; such cells will be predominantly pre-B rather than B cells) and Eμ-myc tumour cells, isolated without further purification from lymph nodes; such preparations may be either pre-B or B lymphoma cells (Adams et al 1985) and could contain significant numbers of other cell types.
-2nd last line p 5. Replace 'tumor development' with Eμ-myc expression.

1st Revision - authors' response 13 May 2019

EMBOR-2019-47987V2 Author's point-by-point response

Referee #1:

In the manuscript the authors undertake a very detailed analysis with a particular emphasis on the early response during LPS stimulation of mature B cells. The authors find that the early response to LPS stimulation involves only a few hundred genes, rather than amplification of the entire transcriptomic signature (amplifier model), as argued by other investigators.

The data and analysis are very well organized and presented. The manuscript does not provide any particularly novel findings regarding the function of Myc, as previous studies, well referenced by the authors, have addressed both the Myc amplifier model and the other findings presented in this study. Nonetheless the data do strongly support the argument against the controversial notion that Myc acts as a global amplifier of transcription in B cell activation. Furthermore, the detailed time-course following LPS stimulation represents a unique resource.

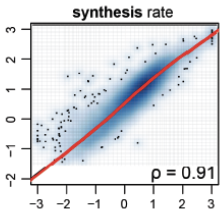
> ***We agree, and wish to highlight the latter aspect brought up by the referee: beyond the debate on the "amplifier model" – which we believe should no longer be at the center of the attention in the field – our work provides an important resource by unraveling the genetic programs regulated by Myc during B-cell stimulation.***

Specific Comments

Figure 1. Figure 1E apparently largely depends on an analysis of RNA synthesis rates that are modeled, but not measured. However, validation of this method appears to be lacking (e.g. comparison of the data derived from this method to a pulse measurement, such as 4sU labeling). Without this, the pulse data cannot be considered rigorous.

> ***There was a misunderstanding here, due to incomplete explanation in our original text and figure:***
>
> ***The "mature RNA" and "precursor RNA" sections of Figure 1E already showed quantitative data: in those columns, the color codes directly reflect the changes in RNA levels as directly measured by RNA-seq, and are not based on modeling. Alongside those data, the figure showed the computationally modeled features (synthesis, processing and degradation rates) that were derived from the data. Most importantly, the RNA-seq data for***

precursor RNA are conclusive as such, and do not necessitate independent confirmation. Indeed, our precursor RNA values were based on measuring intronic and exonic reads in total RNA-seq experiment, which constitute a formal, quantitative measurement and provide a valid alternative to measuring 4sU-labeled RNA. This was demonstrated in an independent manuscript, originally cited as "in preparation": this is now publicly available as a reprint (Furlan et al. 2019 bioRxiv; doi: https://doi.org/10.1101/520155), as cited in the revised text (p. 4) and in the Materials & Methods section (p. 16).



To further illustrate this point, we show here a panel from Furlan et al. 2019 (Fig. S3), which represents the correlation between the estimated synthesis rates as modeled with intronic and exonic reads from total RNA-seq (X axis) or with 4sU labeling (Y axis), following MycER activation in fibroblasts. This demonstrates the high consistency between the two approaches (correlation coefficient: 0.91).

Last but not least, whether using 4sU labeling or total RNA-seq, the deduced rates (synthesis, processing and degradation) are always the result of computational modeling (de Pretis et al. 2017; Furlan et al. 2019). Hence, in this regard, using 4sU labeling would not be more rigorous than RNA-seq.

Figure 2. The authors point out that Myc binds only to pre-existing promoters/enhances that are essentially marked as active. They call this 'open chromatin'. Open chromatin is a somewhat vague term that can involve many epigenetic marks, and there are certainly studies that indicate Myc has some role in this process (Kieffer-Kwon, et. al., 2017). This current study shows no evidence of global chromatin structure change at these early time points. Perhaps a more specific term such as 'active promoters' should be used.

We agree with the referee, and have modified the title of Figure 2, which now reads "Myc widely associates with active promoters and enhancers upon LPS stimulation", instead of "open chromatin". The text referring to this figure was already stating "active promoters and enhancers" (p.5).

As also correctly pointed out by the referee, we did not assess possible additional changes in the distribution/intensity of chromatin marks upon LPS treatment; however, this does not impinge on our conclusion that new Myc binding sites acquired upon stimulation were already marked by H3K4m3/H3K4me1 and H3K27ac in naïve B-cells.

In this context, it is noteworthy that the findings of Kieffer-Kwon et al. 2017, to which the reviewer was referring, provide an important element in favor of an indirect role of Myc in global chromatin decompaction, in the same cellular model used in our work: indeed, chromatin decompaction was shown to occur through ATP-mediated remodeling, and Myc was required the cells to sustain the adequate levels of ATP production. We have added a sentence in this regard in our conclusive paragraph (p. 9).

In Figure 2H, the data are clear that canonical E-boxes are enriched in cells expressing the lowest levels of Myc (0h), as expected for high affinity sites. Yet this is only a relatively minor fraction (~25%) of the signature. In fact, it appears that approximately 40% of the peaks do not contain even degenerate E-boxes yet would be classified as high affinity sites (since they're bound at lowest Myc). The authors should comment on these promoters, since they really don't behave according to the model the authors suggest (text, top of page 6).

While consensus motifs represent the highest affinity binding sites for transcription factors, they are not the sole determinant of DNA binding in vivo. This is particularly true for factors recognizing simple motifs, as the hexameric E-box. To clarify this better, we have added a new figure (Fig. 2I) and completed the text as follows (p. 5): "In line with these observations, E-box-containing sites were the most efficiently bound by Myc in all conditions, with intermediate levels at regions with variant E-boxes [8, 36, 37] and lowest in absence of any E-box motifs (Fig. 2I); note however that these categories showed large ranges and significant overlaps in binding intensities, consistent with the involvement of additional chromatin- and protein-based determinants [38]."

Figure 4. When assessing recruitment of Pol2 vs elongation, the authors make the important point that recruitment of Pol2 to promoters is the factor that has the highest correlation with differentially expressed genes, even more so than the effects on pause-release. Also, subsequent Pol2 elongation steps should also be dependent on recruitment. The authors use an algorithm to model recruitment and subsequent pause-release and elongation steps. The authors should at least mention that the elongation data might be better addressed using more powerful techniques like ProSeq or 4sU-FP-seq (Liang, and Shilatifard., Cell, 18 October 2018) to map Pol2 with high resolution and pulse methodology.

> ***We modified the text (p. 7) as follows: "… Altogether, Myc appears to primarily drive RNAPII recruitment at activated loci [34], with differential contributions of other regulatory steps, in particular pause-release [45] or elongation [46]. Together with recent studies, our work shows that a careful integrative analysis of RNAPII and RNA dynamics [34, 47], ideally complemented by dedicated assays to monitor elongation (e.g. Pro-Seq or 4sU-FP-seq) [46, 48] is needed to unravel the hierarchical contribution of distinct regulatory steps."***

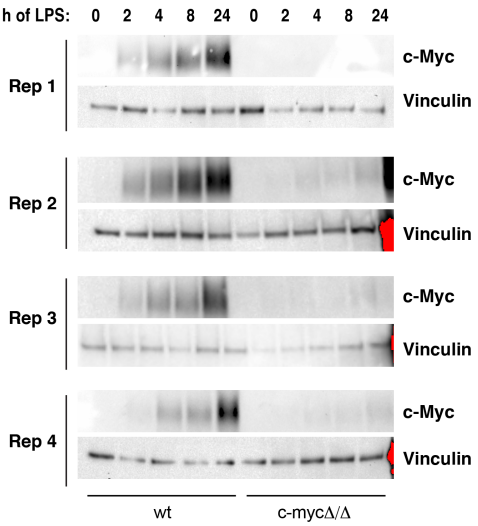
An interesting point here is that Myc is generally lost at genes that are down-regulated by LPS. This is termed a 'passive process', but Myc levels are going up (why aren't these high affinity sites in LPS?). Therefore, the model in which Myc occupies sites based on low/high affinity and Myc levels isn't well explained by this data. This warrants further discussion or investigation as to what is the mechanism involved in this effect (e.g. other histone modifications, chromatin structure, etc.).

> ***As discussed in the text, and as originally described in our previous publication by de Pretis et al (2017), the feature that most accurately predicts the transcriptional response following changes in Myc levels is the relative gain or loss of Myc at each promoter, in a situation in which Myc levels – and hence binding to all promoters – increase globally. For this reason, we defined the "Myc share", in which the Myc signal at each promoter was normalized by the total level of Myc bound to all promoters in each condition.***
>
> ***As written in p.6, "those genes for which the RNA synthesis rate increased upon LPS stimulation also showed the highest gains in Myc binding (i.e. increasing share), while those with a reduced synthesis showed the lowest gains (decreasing share)."***

Minor points

The representative western blot in EV1 isn't very high quality because the Myc bands are so broad. The data would be improved with a higher quality blot.

> ***We must disagree with this assessment, and thus with the associated request to repeat our immunoblots:***
>
> ***In EV1 we reported quantifications derived from 4 different western blots, and provided a single blot as a representative example. All of the blots are shown here for the sake of full illustration. As now explained in the legend, each of those blots was produced with lysates obtained from a pool of 3 mice per genotype.***
>
> ***Even if the sharpness of the bands may in principle be improved, we deem those experiments as fully and formally conclusive: their quantification is not ambiguous, and the impairment of Myc induction in deleted cells is clear in each of the 4 experiments. Furthermore, the fuzziness of protein bands in such immunoblots may owe to the presence of multiple post-translational modifications, which are beyond the scope of our present work. Altogether, we deem it unjustified to repeat these experiments with the mere (and most likely elusive) goal to obtain sharper bands.***



Referee #2:

In this report, Tesi, de Pretis et al. address a basic question - what is the role of the transcription factor Myc in the early hours of B cell activation - and they provide yet another piece of evidence that, instead of being a non-selective transcriptional amplifier, a regulator of cell cycle or DNA replication, Myc recruits RNA polymerase to a well-defined set of genes responsible for RNA metabolism and energy production which prepare the cells for future growth and proliferation.

Their experimental system is simple, Tat-cre mediated deletion of floxed Myc alleles in primary B cells followed by LPS stimulation which they characterize in sophisticated detail by RNAseq and ChIPseq methods and thorough bioinformatic analysis. I particularly appreciate the great emphasis they put to assess the deletion efficiency and identify the extent of Cre-mediated deletion escapees.

The report by Tesi, de Pretis et al. is of interest to a broad scientific community, particularly scientists interested in Myc-mediated gene regulation, but also B cell activation.

This is a solid scientific report which has logical flow. It is well written, although some parts need further/better explanation.

I have 2 comments and minor points I would ask the authors to address:

Comments:
1)
The authors observe a correlation between the gain of Myc binding at the promoters (Myc share) and the increase of RNA synthesis from these promoters. This correlation is the strongest for Myc-dependent genes, although still present at Myc-independent loci (Figure 3 a,b). The discussion of the "at first sight paradoxical" findings and "the notion that promoter activity and Myc binding are mutually dependent" is confusional and appears contradictory to the finding of Myc-dependent and -independent set of genes. The authors should improve the discussion and better explain what they mean.

> ***We thank the referee for this comment, which is indeed an important point in our text. We now modified the text as follows (p. 6): "… albeit paradoxical at first sight, this observation is consistent with the notion that promoter activity and Myc binding are mutually dependent, owing to the interaction of Myc with other chromatin-associated factors, RNAPII and components of the basal transcription machinery [39, 42, 43]. In other words, changes in transcriptional activity at Myc-independent genes are likely to impact in return on transcription factor recruitment."***

2)
To "increase the resolution of the analysis" the authors introduce a clustering approach in Fig 4. From this point on, the flow of the paper becomes very complicated for 3 reasons:

• The aims of the clustering approach are poorly explained
• Clusters (numbers and properties) are poorly described
• The selection criteria of only certain clusters for further analysis are not clearly explained.
The authors should restructure and better explain this section.

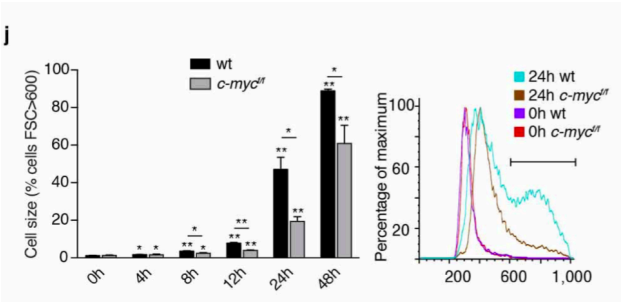
> ***The purpose of clustering here was to go beyond the analysis of average patterns, and to discriminate possible regulatory subsets within the broad classes of Myc-dependent/independent and activated/repressed genes. We have made this clearer in the text by specifying: "To increase the resolution of our analysis and unravel possible regulatory subsets, we clustered LPS-regulated genes on the basis of RNA synthesis rate and RNAPII dynamics …" (p. 7). Independently from the outcome, this type of analysis would be incomplete without such a clustering step.***
>
> ***While no major differences were uncovered (e.g. genes that would be regulated by Myc at totally different stages of the RNAPII cycle), some noteworthy differences did emerge. This***

is why we highlighted specific clusters in the text. For example (p.7), "Remarkably, the largest sets of LPS-activated genes (CL1: Myc-dependent; CL9: Myc-independent), were almost exclusively regulated through RNAPII loading (Fig. 4D-F) while others showed additional effects on other regulatory steps (Appendix Fig. S2, S3). Etc…"

We have displayed all the clusters with the box-plots showing the analysis of RNAPII behavior, in Figures EV4, 5 (now called Appendix Fig. S2, S3). Clearly, though, the point here wasn't to describe every cluster in full detail (which would make the text lengthy and cumbersome). We have made several detail changes to this section, and believe the logic should now be fully understandable.

Minor points:

Figure 1.
B) Provide your own measurement of cell size at 0, 12, 24 h in Myc wt and ko.



We previously measured cell size in the same experimental settings used here and reported these measurements in Sabò et al 2014 (Extended Data Figure 6j: shown here for the sake of illustration). While cell size started to increase around 8h after LPS stimulation, the changes were mainly occurring between 12 and 24h. This was cited in our text (p.4) as follows: "While global RNA levels also increased in a Myc-dependent manner [3, 4], this occurred later (24h, Fig. EV1E) concomitant with increases in bulk RNA synthesis and nuclear size (Fig. 1B), overall cell size [2, 4, 5], as well as S-phase entry (Fig. EV1F) [4]." Since we already reported conclusive data on cell size in exactly the same experimental conditions, we did not repeat these specific measurements. Most importantly, our new data on RNA synthesis and Nuclear size (Fig. 1B) are fully consistent with – and significantly extend – the previous evidence on cell size.

Is "Nuclear Area" the nuclear size?

Yes, obviously: for a spheric object such as a cell nucleus is (in particular in non-polarized cells growing in suspension), the planar area and the volume are directly related. As what we formally measure in our 2D microscopy assay is a planar area (in Pixels), it is formally correct to indicate this as "Nuclear Area (pixels)" on the X axis, and to write "nuclear size" as we did in the text. As pixels are not a metric unit, we did not calculate a volume for each nucleus, but this isn't formally required to draw a conclusion from the data. We have also amended the text as follows"

- ***Materials & Method (p.11) as follows: "Nuclei were detected on the basis of DAPI staining using a Perkin Elmer proprietary algorithm and each nucleus was associated with its planar area (in pixels) and its integrated EU signal."***
- ***In the Figure legend (p.22): "The scatter plots show the nuclear Area as detected in the 2D plane (x axis, in pixels) versus the EU signal (y axis, as arbitrary units, AU) for each single cell identified by DAPI staining …"***

Dots represent individual cells?

Yes, each dot represents a single cell, for which we report Nuclear Area (x-axis) and EU signal (y-axis). The Figure Legend (p. 22) was amended as follows: "The scatter plots show the nuclear Area as detected in the 2D plane (x axis, in pixels) versus the EU signal (y axis, as arbitrary units, AU) for each single cell identified by DAPI staining in c-myc$^{wt/wt}$ and c-myc$^{\Delta/\Delta}$ populations at the indicated time points after LPS treatment."

C) The numbers which should help the reader identify gene subsets are hard to read.

The figure was amended accordingly.

Define the "grey zone" in the text. The different regulatory groups should be clearly defined in the text.

We had already indicated the criteria for Myc-dependent and independent genes, which unambiguously defined those for the "grey zone". To improve clarity, we now rewrote this as follows (p. 4): "We defined Myc-regulated genes as those for which the magnitude of the LPS response was reduced by at least 1.5-fold in c-myc$^{\Delta/\Delta}$ relative to c-myc$^{wt/wt}$ cells […] On the other hand, significant fractions of all mRNAs showed Myc-independent up- or down-regulation by LPS (altered ≤1.15 fold in c-myc$^{\Delta/\Delta}$ relative to c-myc$^{wt/wt}$ cells; groups 5, 6; Fig. 1C, D, Supplementary Table 1, 2). As expected, other genes fell in what we will qualify as the "grey zone", with intermediate levels of Myc-dependency (Fig. 1C)."

Figure EV1
Is the cell survival of Myc wt and ko cells comparable at different time points upon TAT-cre transduction?

We have analyzed cell survival in Myc wt and ko cells at different time points after LPS stimulation through measurement of Caspase 3/7 activity, which is now shown in Fig. EV1I. As written in the text (p. 4), "the apoptotic response observable at late time-points (72h onwards) was also reduced in c-myc$^{\Delta/\Delta}$ cells."

Importantly, these data also show that, at early time points, apoptosis was low in these wt and ko cultures (all treated with tat-CRE). In this regard, it is noteworthy here that B-cell populations were isolated in exactly the same manner from c-myc$^{wt/wt}$ and c-myc$^{f/f}$ animals, and exposed to tat-Cre: this allowed us to analyse of the effects of deletion in c-myc$^{f/f}$ cells (indicated as c-myc$^{\Delta/\Delta}$) by direct comparison with identically treated c-myc$^{wt/wt}$ cells, without the need to correct for non-specific / toxic effects of tat-Cre.

D) To better understand the binding dynamics of Myc, show ChIP qPCR for 2, 4 and 8 hours in Myc ko and wt cells.

ChIP-seq data provide a much more rigorous indication of binding dynamics at all promoters and distal sites, compared to ChIP-qPCR on few targets, considering that binding levels are highly variable between promoters (Fig. 2G, I). Fig. 2I is new, and allows to visualize the binding dynamics to different categories of sites (promoters vs. distal, with or without E-boxes) in wt cells.

The loss of Myc binding in ko cells was clearly indicated by our preliminary ChIP-qPCR analysis in Fig. EV1D, consistent with the loss of the protein and the RNA. Having formally made the point, further analysis of Myc binding by ChIP was unnecessary, and has not been pursued.

Does Ncl contain E boxes?

The mouse Ncl gene contains 5 E-boxes in intron 1 (see Fig. 2 of Greasley et al. 2000 Nuc Ac Res 28, 446-453), as now specified in the legend to Fig. EV1D.

Can you confirm higher binding of Myc on E-box promoters as compared to promoters that do not contain E-boxes by ChIP qPCR?

Confirming higher Myc binding to E-box-containing promoters compared to the ones devoid of E-boxes could be done by qPCR but would be very biased and non-representative of the variability found at a genome-wide level, even within each of those categories. We thus deem it much more reliable to compare the range of ChIP-seq signals among the thousands of binding sites that can be mapped by ChIP-seq, as now visualized in Fig. 2I and in the text (p. 5): "In line with these observations, E-box-containing sites were the most efficiently bound by Myc in all conditions, with intermediate levels at regions with variant E-boxes [8, 36, 37] and lowest in absence of any E-box motifs (Fig. 2I); note

however that these categories showed large ranges and significant overlaps in binding intensities, consistent with the involvement of additional chromatin- and protein-based determinants [38].”

Figure 2
B) The CpG mentioned in the figure legend is not found in the figure.

Removed from the figure legend

G) This figure needs better explanation in text and figure legend. What is the color code and rows?

We have modified the figure to make it more intuitive, as well as the legend: this connects it directly to the conclusions given in the text.

Figure 3
B) This graph is not easily comprehensible without a brief explanation in the text. Moreover, the axis, sensitivity and specificity shall be explained in the figure legend. What is the grey line?

A receiver operating characteristic (ROC) curve, as shown in Fig. 3B, is a common standard in representing the validity of a predictor (here, Myc share) in discriminating between variables: as such, we do not deem is reasonable to have to extend the text in explaining what this is. On the other hand, we have done this is the figure legend, as follows:

“The ability of the Myc share in predicting the transcriptional outcome can be represented in terms of a Receiver operating characteristic (ROC) curve, which represents the Sensitivity (true positive rate) and Specificity (true negative rate) of a predictor (i.e. Myc share) in discriminating between two classes (i.e. up- and down-regulated genes), using different thresholds. The largest the area under the curve (AUC), the largest is the predictive power, in a scale from 0.5 (random classification: grey line) to 1 (perfect predictor). For each system, the dot corresponds to the variation of Myc at which promoters begin increasing their share of Myc binding.”

Dependent and independent stands for Myc-dependent and -independent?

Yes. This has been clarified in the figure.

Figure EV 3 C, D); Figure 4 F-H); Figure EV 4, 5 missing statistics.

The figure was amended accordingly.

Figure EV6 A short explanation is needed in text.

The text has been changed as follows: “Most importantly in this context, the differential occurrence of consensus binding motifs suggests that other transcription factors, such as E2F or NFY, may have predominant roles – or be redundant with Myc [49] – in regulating Myc-independent genes (Fig. EV4). “

Minor suggested text edits:
p.6
...in either B-cells (Guccione, Martinato et al., 2006, Lin et al., 2012, Nie et al., 2012, Sabò et al., 2014) or other cell types ...

corrected

p.7
... Myc drove rapid and selective activation of high-affinity promoters, most frequently associated with the presence of the

E-box binding motif.

corrected

p.9
...we have identified approximately 650 genes that were induced in a Myc-dependent manner within 4-8h following B cell stimulation by LPS, and an additional group of ca. 1100 regulated by Myc with modest quantitative...

corrected

... Interestingly, the Myc-dependent program identified here in activated B-cells was constitutively deregulated during lymphomagenesis in Eμ-myc transgenic mice (Sabò et al., 2014)...

corrected

Referee #3:

The study by Tesi et al aims to delineate early Myc-dependent gene expression profiles during B-cell activation. To do so, they compared c-myc+/+ and c-mycf/f primary B cells stimulated by LPS following exposure to tat-Cre recombinase. A major conclusion is that, during LPS-driven B-cell activation, MYC drives rapid and selective activation of several hundred high affinity promoters, through further RNAPII recruitment, and that the genes affected encode proteins involved in RNA biology, energy production and anabolic pathways. By comparing profiles obtained after LPS stimulation with those they have previously reported for pre-malignant and malignant B lymphoid cells from Eμ-myc mice (Sabo et al Nature 2014), they conclude that the same genes are activated in B lineage cells driven by oncogenic high levels of MYC. They conclude that MYC has no direct impact on global transcriptional activity, contrary to the amplifier model of MYC activity.

The paper deals with an important and still-controversial biological issue: the mechanism of action of MYC. In general it is well-written, succinctly presented and provides original data that will be of interest to the many researchers studying MYC. I recommend publication, contingent on consideration of the following comments and appropriate minor revision.

1. Abstract line 5-6. Description of protocol is misleading. Change to, for example, 'We addressed this in LPS-stimulated mouse B-cells, by comparing WT versus c-myc-deleted cells'.

We re-wrote this sentence as follows: "We addressed this issue by profiling the response to LPS stimulation in wild-type and c-myc-deleted primary mouse B-cells."

2. To my mind, insufficient attention is paid in the paper to characterising the cell populations used for LPS treatment and RNA profiling. FACS profiles should be provided showing the viability, cell size and immunophenotype before and after treatment with tat-Cre.

We must disagree with this assessment, for the following reasons:

1. ***We should emphasize that B-cell populations were isolated in exactly the same manner from c-myc$^{wt/wt}$ and c-myc$^{f/f}$ animals, and both exposed to tat-Cre: this allowed us to analyse of the effects of deletion in c-myc$^{f/f}$ cells (indicated as c-myc$^{\Delta/\Delta}$) by direct comparison with identically treated c-myc$^{wt/wt}$ cells, without the need to correct for non-specific / toxic effects of tat-Cre.***
2. ***Recombinant tat-CRE may in principle have been contaminated by bacterially-derived components (LPS and others), which may have interfered with our subsequent analysis of the LPS response. In order to avert this potential complication, we ran preliminary experiments, now shown in Appendix Fig. S1A, in which we treated primary B-cells with tat-Cre (1h), washed the cells and monitored the expression of 2 LPS-responsive genes (Junb and Ikba) by RT-PCR at different times after treatment (0,1,12h). Indeed, we observed activation of those genes by tat-Cre (and by LPS as positive control), but this was invariably down to background (i.e. the***

same as in untreated cells) after 12h, at which time we started our LPS time-course experiments. In addition, we verified that the pretreatment with tat-CRE was not affecting the response to LPS Appendix Fig. S1B.

3. ***We also verified that tat-CRE treatment per se was not acting as a mitogenic stimulus by performing growth curves of B cells treated with either tat-CRE alone, LPS alone, or tat-CRE followed by LPS (Appendix Fig. S1C and part in EV1G). The results showed that tat-CRE (a.) did not induce cell proliferation by itself, and (b.) impaired the proliferative response to LPS selectively in c-myc$^{f/f}$, as opposed to c-myc$^{wt/wt}$ cells. These additional controls have now been cited in Material and Method at p.10 as "Effects of Tat-Cre treatment on wild type B cells activation or proliferation were shown in Appendix Fig. S1."***
4. ***We have analyzed cell survival in c-myc$^{wt/wt}$ and c-myc$^{\Delta/\Delta}$ cells at different time points after LPS stimulation through measurement of Caspase 3/7 activity, which is now shown in Fig. EV1I. As written in the text (p. 4), "the apoptotic response observable at late time-points (72h onwards) was also reduced in c-myc$^{\Delta/\Delta}$ cells." Importantly, these data also show that, at early time points, apoptosis was low in these c-myc$^{wt/wt}$ and c-myc$^{\Delta/\Delta}$ cultures (all treated with tat-CRE).***

Altogether, the above observations control for non-specific effects of tat-CRE, and ensure that we are studying the effect of c-myc deletion in B-cells. In this situation, we do not see what measurement of viability, cell size or immunophenotype before and after treatment with tat-Cre, as requested by the reviewer, would add to our work.

I would also like to see evidence of c-myc deletion by Southern blot or PCR.

We confirmed Myc deletion by qPCR on genomic DNA (EV1A), RT-qPCR on mRNA (EV1B) and western blot (EV1C), which constitutes a compelling and fully conclusive set of data. In this regard, it is noteworthy here that referee #2 wrote "I particularly appreciate the great emphasis they put to assess the deletion efficiency and identify the extent of Cre-mediated deletion escapees."

Objectively, we deem that no additional confirmation is required to formally conclude that the gene has been deleted. Southern blotting, in particular, would be superfluous in this setting, since the same point is made by our qPCR analysis on genomic DNA.

Was the Cre protein used the His-Tat-NLS-Cre described by Peitz et al?

Yes, we have now specified this in the Materials & Methods section.

3. How many independent batches of cells were analysed?

The number of biological replicates (independent batches of cells) for each experiment has been specified in the corresponding figure legend as n=x.

4. p4 para 2
- Line 8. The case is overstated - only Ncl and Pus 7 expression are convincingly Myc-dependent. Modify sentence accordingly.

We agree, and have removed the third gene tested (Smyd2): while this gene was identified as Myc-dpendent in Fibroblasts (Perna et al.), its response was moderate in B-cells. As a consequence, while still Myc-dependent, this remained below statistical significance at most time-points. As we only used these tests as preliminary criteria for our RNA-seq profiles, and as we are making no specific point about Smyd2, we have removed it from the figure.

- indicate in text that the B cells are isolated from spleen

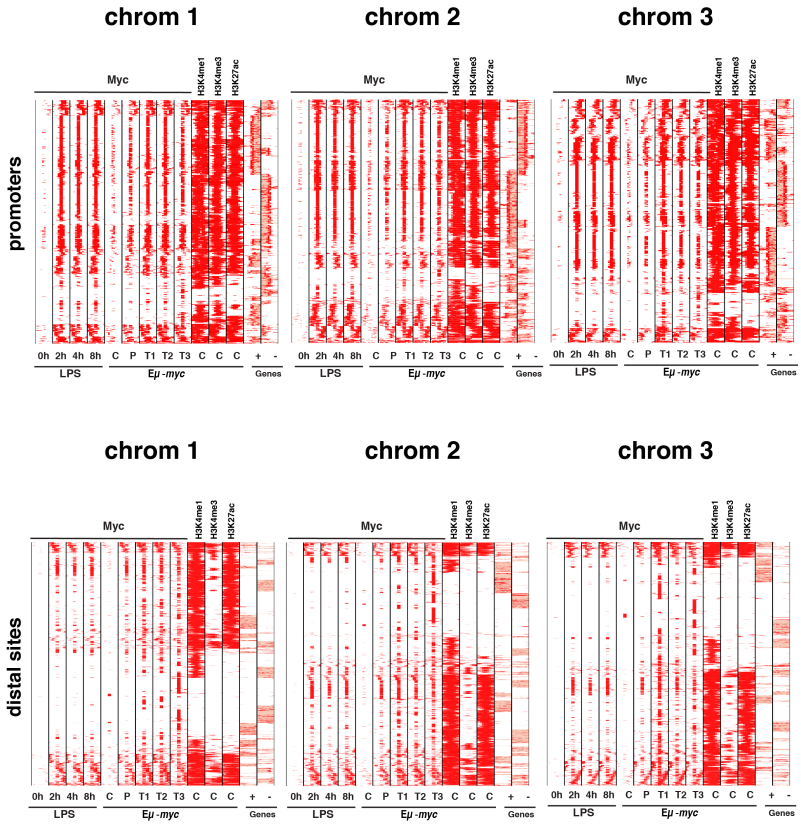
We have now specified this in the text (p.4), as follows: "Freshly purified c-myc$^{f/f}$ and control c-myc$^{wt/wt}$ <u>splenic</u> B-cells were treated…"

5. Figure 2
- 2a: description in text and legend is inadequate to enable the reader to understand this diagram.

We have modified the figure and figure legend as following: "Overlap between Myc ChIP-seq peaks. For each time-point (indicated on the bottom), the percentage of peaks overlapping over ≥1 bp with any other time-point (Reference sample) is reported. "

-2b: Why were chromosome 1 Myc-binding promoters selected? Were similar results obtained for sites on other chromosomes?

We selected chromosome 1 as representative of the rest of the genome. To validate this choice, we show two other chromosomes below, as additional examples. The concepts gathered from those panels are exactly the same: we deem it thus correct to show chromosome 1 (the largest in the genome) as representative of the variations in Myc-binding across the various samples.



In any case, I would be cautious re the comparison of the LPS data obtained here for splenic B cells isolated by magnetic bead depletion of non-B cells versus those reported in Sabo et al (2014) for control WT B-cells (C; enriched by binding to B220+ beads), premalignant splenic cells from age-matched (6-8wk) Eμ-myc mice (P; enriched by binding to B220+ beads; such cells will be predominantly pre-B rather than B cells) and Eμ-myc tumour cells, isolated without further purification from lymph nodes; such preparations may be either pre-B or B lymphoma cells (Adams et al 1985) and could contain significant numbers of other cell types.

We agree with the referee that we may have alterations in the differentiation state of B cells in the pre-tumoral Eμ-myc samples, as well as infiltrating cells in the tumor samples. Yet, if anything, this makes the high similarity of the Myc-binding profiles in LPS-treated

and Eμ-myc samples even more remarkable. With all due caution, we believe that this allows us to push forward the concepts made in our text.

-2nd last line p 5. Replace 'tumor development' with Eμ-myc expression.

Corrected

2nd Editorial Decision 13 June 2019

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, all referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address the below minor/editorial points:

REFEREE REPORTS

Referee #1:

The authors have adequately responses to all my concerns and recommend publication of the paper. I would ask the authors to consider two points:

1. Relating to comments on fig. 1 - the auhtors' explanation is acceptable, though I disagree with the last statement, and argue that a 4sU or similar strategy measurement would provide a better set of base data for modeling synthesis rates.
2. In response to reviewer 2, the authors have introduced the sentence "In other words, changes in transcriptional activity at Myc-independent genes are likely to impact in return on transcription factor recruitment." This sentence doesn't really make sense. Are they arguing that Myc depletes vague 'chromatin associated factors' to regulate promoters that are 'Myc independent'. This appears to be a muddled point as currently discussed in the paper, as I'm not sure what the authors are trying to say.

Referee #2:

The authors have satisfactorily addressed all points raised by the referees and substantially improved the paper.

Referee #3:

The authors have satisfactorily addressed most of the points of criticism I raised. For the remainder, although I do not fully accept their point of view, logical arguments were put forward and I do not believe our differences merit withholding approval for publication.

2nd Revision - authors' response 18 June 2019

Referee #1:

1. Relating to comments on fig. 1 - the auhtors' explanation is acceptable, though I disagree with the last statement, and argue that a 4sU or similar strategy measurement would provide a better set of base data for modeling synthesis rates.

There is no question that 4SU labeling would provide a direct experimental measurement of newly synthesized RNA. However, Furlan et al. (ref. 35) showed that the use of intronic and exonic reads in total RNA-seq data provides a reliable surrogate of such direct measurement. This is the basis of the analysis provided in our work, as specified in our text:

"Besides mature mRNAs, we sought to measure the accumulation of unspliced precursors along the time-course, and to use these data for computational modeling of RNA synthesis, processing and degradation rates: while originally based on metabolic labeling of newly synthesized RNA [34], this can readily be achieved with intronic and exonic reads in total RNA-seq data (Fig. 1E) [35]."

2. In response to reviewer 2, the authors have introduced the sentence "In other words, changes in transcriptional activity at Myc-independent genes are likely to impact in return on transcription factor recruitment." This sentence doesn't really make sense. Are they arguing that Myc depletes vague 'chromatin associated factors' to regulate promoters that are 'Myc independent'. This appears to be a muddled point as currently discussed in the paper, as I'm not sure what the authors are trying to say.

By "likely to impact in return on transcription factor recruitment" we simply refereed to the recruitment of MYC itself, and not of some undefined transcription factor. We have changed the text accordingly.

3rd Editorial Decision 27 June 2019

Thank you for submitting your revised manuscript. I have now taken a look at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on the very nice work!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Bruno Amati
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-47987

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?	p18 methods
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	p18 methods
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	p9 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.	p9 methods
For animal studies, include a statement about randomization even if no randomization was used.	p9 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.	p9 methods
4.b. For animal studies, include a statement about blinding even if no blinding was done	p9 methods
5. For every figure, are statistical tests justified as appropriate?	p18 methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	p18 methods
Is there an estimate of variation within each group of data?	p18 methods
Is the variance similar between the groups that are being statistically compared?	p18 methods

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).	p10 and p12 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.	N/A

* 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.	p9 methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	p10 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.	p10 methods

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	p18 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).	p18 methods
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).	p18 methods
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.	p18 methods

G- Dual use research of concern

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