Author’s response to reviews

Title: Myc suppression of Nfkb2 accelerates lymphomagenesis

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Author’s response to reviews: see over
To:
Dr Philippe Juin
Associate Editor
BMC Cancer

Munich, May 21st, 2010

Re: Resubmission of MS1124431948347814 “Myc suppression of Nfkb2 accelerates lymphomagenesis” by Keller, Huber et al.

Dear Dr. Juin,

Please find enclosed the submission of our revised article “Myc suppression of Nfkb2 accelerates lymphomagenesis” (MS1124431948347814) by Jürgen Huber, Jonas A. Nilsson, Mohammad Fallahi, Mark A. Hall, Christian Peschel, John L. Cleveland, and myself for consideration as a Research Article.

I would like to thank you for your invitation to submit a revised article and for also allowing an extension for our resubmission, so we could properly address the Reviewers’ constructive criticisms and helpful suggestions.

We were very pleased with the overall appreciation of our study by the two Reviewers and we have addressed, wherever feasible, their concerns. The comments of the Referees are copied below (in Times-New Roman font and flanked by quotations). Our point-by-point responses to their comments and suggestions are provided in bold font immediately below, and are as follows:

Reviewer: Benjamin Barré

“1. The authors suggest the overexpression of Myc in Eu-Myc B220+ (fig1) is responsible of the inhibition of Rel/NF-kB subunit, but in Eu-Myc lymphomas, there is not variation of myc expression (fig1). The authors should clarify why there is a stronger inhibition of Rel/NF-kB subunit expressions without variation of Myc expression in these lymphomas. It might be interesting to analyze the expression of Myc partners such as Mad, Miz-1 or Max.”

We and others have shown that the Rel/NF-kappaB pathway is suppressed in Myc-expressing cells (Keller et al., 2005, Oncogene 24:6231-6240; Schlee et al., 2007 Int J Cancer 120:1387-1395; Dave et al., 2006, N Engl J Med 354:2431-
Our studies presented herein suggest that Myc-mediated suppression of \textit{Nfkb2} promotes lymphomagenesis, but we cannot conclude from our data that once the disease is established that other mechanisms that suppress \textit{Nfkb2} are not involved. To our knowledge, no study has shown an intra-lymphoma correlation between \textit{MYC} and \textit{NFKB/REL} transcript levels. Furthermore, once lymphomas are established in Eµ-Myc transgenics additional genetic lesions may occur.

As suggested by both Reviewers we have reanalyzed the Affymetrix data and we have included the suggested control genes into Figure 1: \textit{Mad} (\textit{Mxd1}), \textit{Miz1} (\textit{Zbtb17}), \textit{Max}, p15 (\textit{Cdkn2b}) and p21 (\textit{Cdkn1a}).

“2. The fig2a & b clearly show a relationship between Myc expression and NF-kB2 inhibition in bone marrow and spleen cells. However, the same kind of experiments should be performed in Eu-Myc lymphomas and burkitt lymphomas to establish the same correlation in fig2c-e (mRNA and protein levels of Myc and NF-kB2 in these both lymphomas).”

Regarding Myc mRNA and protein levels in Eµ-Myc lymphoma and in human Burkitt lymphoma (BL) these data have been published by our group and by others and these studies are cited in the manuscript. We no longer have access to necessary amounts of the primary BL samples that would be required to determine NF-kappaB2 protein by western blots.

“3. The fig3c is poor quality picture compared to the previous western blots (fig 2b & 2d) and show discordance with the data from the fig 3b. On the 4th lane (-T 6h) of the Fig3c, the expression level of NF-kB2 is higher than on the 2nd lane (+T+E 6h), showing the inverse of the authors’ hypothesis. The authors need to clarify this point.”

The principle finding that is clearly shown in Figure 3b is that \textit{Nfkb2} mRNA inversely correlates with c-Myc transcripts in a Dox-inducible Myc transgene model (human P493-6 B cells). We agree with the Reviewer that the original Figure 3c was difficult to interpret, particularly since post-translational changes in p100 and p52 can occur. To simplify this for the reader we deleted Figure 3c in the revised manuscript.

“4. The authors speculate that Myc might regulate the inhibition of NF-kB2 expression like on the p21 gene. To further establish the role of Myc on NF-kB2 gene, luciferase plasmids with a mutated INR site on NF-kB2 promoter should be transfected into cells and reporter gene assay should be performed to determinate a direct role of Myc on this gene. Moreover, to demonstrate that Myc inhibits the constitutive Miz-1-mediated NF-kB2 expression, the authors should perform ChIP assay (or EMSA). If the hypothesis is correct, ChIP assay will show a constitutive recruitment of Miz-1 on NF-kB2 promoter in wt B cell while the Eu-myc B cell will recruit Miz-1 and Myc on this promoter.”

As stated in our original manuscript, we agree with the Referee that our data do not exclude Miz-1 binding to the endogenous \textit{Nfkb2} promoter. In the short time available for resubmission we were unable to establish a Miz-1 ChIP assay to address this issue. We thus respectfully request that our statement that our
data provide indirect evidence for Miz-1 involvement remain in the revised manuscript. Defining the precise mechanism of Nfkb2 transcriptional control issue will be the subject of future studies.

Minor Essential Revisions

“1. statistics need to be done for the fig3a.”

As requested, we now provide statistical analyses of the data presented in Fig. 3a in the figure legend.

“2. error bars and statistics need to be done for the additional figure.”

We have repeated the MEF experiments and have shown activation of Myc suppresses Nfkb2 mRNA levels (Fig. 3a). The established Myc target gene E2f1 and the Myc suppression target p27 were included and statistical assessments are shown (see Additional Figure 1).

“3. p21 or p15 expression might be good control for the fig1 & 2.”

As suggested by the Reviewer we have now included p15 (Cdkn2b) and p21 (Cdkn1a) in our RNA expression analysis (Figure 1, Affymetrix data). Furthermore, we have now included data on p21 mRNA in Figure 2a.

“4. The number of animals used to realize the fig4 is a good quality control and need to be mentioned.”

As requested by the Reviewer we have included the number of animals assessed in our revised manuscript (page 5). The final tally of the two cohorts were 80 Eµ-Myc;Nfkb2+/+ and 44 Eµ-Myc;Nfkb2−/− mice.

Reviewer: Jean Feuillard

In this manuscript, authors report on the effect of Myc on p100/p52 expression and on the effect of p52 on Myc related oncogenesis. It is demonstrated an anti-oncogenic role of p52 in the context of Myc driven lymphomagenesis. It is also shown that c-Myc counteract the anti-oncogenic effect of p52 at the transcriptional level, repressing the luciferase activity under the control of the p52 promoter. This paper is interesting, reporting novel findings. Only technical comments are made to improve the quality of the results.

“- Figure 1: to see differences is not sufficient. Authors should show a clustering of their gene expression profile (or any other adequate method) to demonstrate with objectivity the xistence of the 3 categories”

We agree with the Reviewer that clustering might be superior as compared to just showing differences and have therefore included a cluster analysis in
revised Figure 1. As expected, the control B cells from the 5 wild type mice fall into one cluster, while the pre-cancerous and lymphoma B cells each form unique clusters. As suggested by Reviewer #1 and Reviewer #2 we included some relevant control genes into the analysis (please see our response above).

“- Figure 2: authors should explain why they see a third band between p100 and p52 (panel b and d). They should indicate the position of the weight markers to ensure the reader that the right bands are identified”

We included B-cell lysates from Nfkb2−/− mice in Figure 2d (lane 4) to confirm that proteins identified in our western blots were indeed p100 and p52. Thus, we believe that labeling the bands is not necessary. The third protein noted by the Reviewer (around 70kDa) is also present in lane 4 – Nfkb2−/− B-cell lysates – thus, this represents a nonspecific band detected with this antibody, and this protein is likely more abundant in Eµ-Myc lymphomas. The lysates from Eµ-Myc lymphomas were from frozen total tumors, and thus they do contain cell types in addition to B220+ B cells that can confound analyses. We have previously observed this phenomenon assessing other proteins in Eµ-Myc lymphomas versus B220-purified B cells.

“- Figure 3: western blot of p100 and p52 should not be cut. Both proteins should be shown on the same gel. This blot could be of better quality. Weight markers should be indicated”

Please see our response to point 3., Reviewer #1.

“Moreover, careful editing of the manuscript should be done since some typographical mistakes are scattered along the text.”

We thank the Reviewer for noting these inaccuracies. The manuscript has been edited to remove all typographical errors.

“Altogether, this paper deserves to be published after these proposed modifications being performed.”

We thank the Reviewer for this recommendation.
As I noted in my original correspondence, and as underscored by the comments of the Reviewers, we strongly feel that our manuscript is perfectly suited for publication in *BMC Cancer*. Our findings represent a significant advance for the field as they establish the non-canonical NF-κB pathway as a relevant *in vivo* Myc target in lymphoma development.

The format of the document follows all of the requirements as listed in the guide for authors, including length restrictions of the Abstract (193 words) and the Manuscript (3382 words, excluding Legends and References). The submission includes a Cover letter, a Word file with the text, four (4) Figures and one (1) Additional data file (Additional Figure).

The manuscript has been approved by all of the authors, this work has not been previously reported, and it is not under consideration for publication elsewhere.

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I thank you very much for your consideration of our revised article and I hope that the manuscript is now acceptable for publication in *BMC Cancer*.

Sincerely,

Ulrich Keller