Author's response to reviews

Title: Identification of synthetic lethality of PRKDC in MYC-dependent human cancers by pooled shRNA screening

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Author's response to reviews: see over
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Editor, BMC Cancer
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Dear Editor-

We appreciate the opportunity to resubmit our revised manuscript (MS# 3239736691239805) entitled “Identification of synthetic lethality of PRKDC in MYC-dependent human cancers by pooled shRNA screening” by Zhou, et al. We also would like to thank the reviewers for their comments and suggestions as we feel they substantially strengthened our revised manuscript.

In this manuscript, from a pooled shRNA library screening, we identified and confirmed PRKDC as a novel synthetic lethal target in MYC-overexpressing cancer cells. We demonstrated that knockdown of PRKDC expression in MYC-overexpressing cells led to a significant reduction of MYC-dependent cell proliferation. Additionally, we observed that PRKDC can modulate MYC mRNA and protein expression levels.

In the revised version, we further strengthened the mechanistic studies of PRKDC in MYC-driven cancer. Our data demonstrated that the inhibition of PRKDC in MYC-overexpressing cells further exacerbated DNA damage as measured by γH2AX. Our study provided evidence for MYC-overexpressing cancer cells to rely more on DNA repair machinery where PRKDC plays a vital role. Furthermore, we also showed that proteasome inhibition cannot completely rescue PRDKC inhibitors’ effect on MYC protein abundance, suggesting that PRDKC functioned at both MYC protein and mRNA levels. We also provided more supporting data to reinforce the claims in the manuscript, including western blot analysis for PRKDC knockdown, consistent effects from a second PRKDC inhibitor, and so on.

Below we have included a point-by-point response to the comments from the reviewers (see underlined text).

We thank you for your comments and input and look forward to your decision.

Sincerely,

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Point-by-point response to reviewers specific comments (see underlined text).

Reviewer 1 Specific Points:

Major Compulsory Revisions
1. Figure 5 does not bring much novelty to what is already known about Myc overexpression and DNA damage. Although the authors’ speculated role of PRKDC in this relationship is logical, I think it is crucial to demonstrate it. If PRKDC is essential for repairing induced DNA damage in Myc-overexpressing cells, this should be reflected by increased levels of gamma-H2AX in cells expressing shRNAs against PRKDC, or treated with a PRKDC inhibitor.

We performed the relevant experiments as suggested and updated Figure 5. When we treated c-MYC, N-MYC or N-MYC overexpressing cells with or without a PRKDC inhibitor, we noted increased levels of \( \gamma \text{H2AX} \) upon PRKDC inhibition.

2. The authors propose that PRKDC is important for Myc mRNA abundance (Fig. 4B), using a PRKDC inhibitor. Authors should confirm reduced Myc mRNA levels when knocking down PRKDC in their stable inducible shRNA lines (Fig. 4C).

We used inducible shRNAs against PRKDC (Fig. 4C), and observed reduction of MYC mRNA using shPRKDC-3, and a minimal effect was observed using shPRKDC-5. It is possible that the residual amount of PRDKC after knockdown could cause the difference between different shRNAs or PRKDC inhibitors.

3. The authors further propose that PRKDC is important for Myc protein stability, but fail to directly address protein stability anywhere in the manuscript. In order to keep this claim in their model, authors should at least rescue the decreased Myc protein levels following PRKDC inhibition (Fig. 4A), through proteasome inhibition (e.g. MG132). Alternatively, they can address Myc protein stability by performing cycloheximide (CHX) chases +/- inhibition of PRKDC. These are rather straight
We performed the suggested experiments to examine MYC protein abundance following PRKDC inhibition, with or without proteasome inhibitor treatment. The PRKDC effect on MYC abundance still exists in the presence of proteasome inhibition. It is consistent with the MYC mRNA reduction data upon PRKDC inhibition, suggesting PRKDC is involved in the regulation of both MYC mRNA and protein levels. We updated Figure 4, and revised the main text and discussion accordingly.

- Minor Essential Revision
1. The authors should indicate in the legend of Fig. 3D-G (left panels) at what time PRKDC knockdown is monitored.

PRKDC knockdown is monitored 4 days after Dox induction.

- Discretionary Revisions
1. In Fig. 3D-G (right panels) it is not clear why viability is assayed by different lengths and time-points for separate cell lines (e.g. 1, 3, 5 days vs. 1, 3, 6 days vs. 0, 2, 4 days). If authors have the data available, it would be better to present this in a consistent way.

The time points were chosen in part due to different growth rates of the cell lines when scheduling the experiments. We agree that it would be more consistent to present the data with the same time points, but unfortunately we do not have additional data points. Nevertheless, we believe that the slight differences in time points will not change the conclusions of the experiments.

Reviewer 2 Specific Points:

Specific concerns:
1. In Figure 2, the knockdown of PRKDC using various shRNAs was shown using RT-PCR in 293T cells. However, subsequent experiments were performed in WI-38 (Figure 2B) and various cancer cell lines (Figures 3 and 4). PRKDC knockdown should be confirmed for all cell lines used for the experiments. Furthermore, Western blot analysis of the knockdown should be shown, either in addition to or in lieu of the RT-PCR data (it does appear that the authors have a working antibody for this protein).

We have updated Figures 2 and 3, showed the PRKDC knockdown data in WI-38 cells and cancer cell lines.

2. The result of the screen is summarized in Figure 1. However, the raw data for the screen should be included as a supplementary. An explanation of how the data analysis was performed and how the heatmap was generated would be helpful. For example, how were the 18 shRNAs used for the heatmap chosen? What cut-off points were used for determination of significance and false discovery rates?
The raw data for the screen is now provided in Supplementary Table S1. The L-MYC1-expressing Day1 samples were required to have a minimal normalized read of 500 counts. The gene list was sorted according to the normalized reads of L-MYC1-expressing Day14 samples. Top 9 genes from this table with <3-fold reduction in control Day1/Day14 samples were selected in the top panel of the heatmap. 9 genes with >3000 reads in L-MYC1 overexpressing Day14 samples were included as the controls in the bottom panel of the heatmap.

3. The authors claim that PRKDC knockdown in Myc-overexpressing cells decreased cell viability. However, they show this by measuring cell proliferation using the CellTiter Glo assay. Using this assay, it is not entirely clear whether the PRKDC suppression increases cell death or causes the cells to grow slower. These results should be complemented by including assays specific for measuring cell death, such as PI or AnnexinV staining.

We have performed Annexin V staining in the cells with or without PRKDC knockdown in SW1271 (control line) and H209 (L-MYC high) cell lines. Stably infected SW1271 and H209 cells were treated with or without Dox for 5 days, and Annexin V staining was performed. As shown in the figure below, we did not see significant apoptosis upon inducible knockdown of PRKDC, suggesting that PRKDC’s effect on cell growth in MYC high cell lines were mainly through cell proliferation, and not through apoptosis.

4. The authors show that Myc overexpression led to an increase in double-stranded breaks, and concluded that this leads to a dependence on DNA repair machinery for cancer cell survival and is responsible for the synthetic lethal relationship between Myc-overexpression and PRKDC inhibition. While this is a reasonable hypothesis, more concrete data needs to be shown to support this mechanism. In the cell lines expressing empty vector, c-Myc, N-Myc, or L-Myc, does knockdown or inhibition of PRKDC cause a further increase in #H2AX compared to the basal levels shown in Figure 5? Does overexpression of PRKDC rescue this increase?
We performed the relevant experiments and updated Figure 5. When we treated c-MYC, N-MYC or L-MYC overexpressing cells with or without PRKDC inhibitor, we noted increased levels of γH2AX upon PRKDC inhibition.

5. With regards to the decrease in Myc levels upon PRKDC inhibition, a description of oncogene addition and how this may contribute to the decrease in viability of Myc-expressing cancer cell lines should be included in the Discussion.

We have updated the Discussion in the manuscript. MYC overexpression in cells leads to oncogenesis. MYC reduction through PRKDC inhibition provides one potential mechanism to target MYC-driven cancer.

Reviewer 3 Specific Points:

- Major Compulsory Revisions
Figure 1. In this figure a heat-map representing some of kinases are shown. To get a fair chance to validate the screen, the whole dataset should be shown. A supplemental table could be uploaded if a graphical representation is not clear.

We included the whole data set in Supplementary Table S1. The concern here is that:

a) although CDK2 and GSK3b are identified, others, well-established kinases such as CSKN1A, CHEK1 and AURKB are not. Discuss or explain why.

This pooled shRNA library was designed in the hope to best knockdown target genes. However, due to resource limitation, we do not have knockdown data for each individual shRNA in the given cell lines. It is highly possible that the failure to identify all previously published MYC synthetic lethal targets as top hits is due to insufficient knockdown of corresponding shRNAs. We view this screen as an outlier screen; any negative data would not be viewed as true negatives. And this will not affect the positive confirmed hits identified from the screen.

b) what is the coverage of the library? Were there reads from all plasmids? We have 1279 shRNAs for 486 human kinase genes, approximately 3 shRNAs per gene. There were reads for 1048 (82%) shRNAs.

- Minor Essential Revisions
Figure 2-3. Adequate experimentation demonstrating that DNA-PK inhibition results in suppression of proliferation of Myc-overexpressing cells. If growth rates are higher of Myc-expressing cells then the effect may be related to that. Please show growth curves of cells expressing vector control, L-Myc-1 and L-Myc-2.

We have checked the growth curves of cells expressing vector control, L-MYC-1 and L-MYC-2. The growth rates were very similar, and we included the data in Supplementary Figure S2.
Figure 4. Very interesting result. Please show Myc protein levels of lymphoma cells treated with the second DNA-PK inhibitor.

We have repeated the Figure 4 experiment with a second DNA-PK inhibitor (NU-7441), and observed a similar effect. The data were included in Supplementary Figure S3.