EMBO reports

# N-glycosylation of PD-1 promotes binding of camrelizumab

Kefang Liu, Shuguang Tan, Wanjun Jin, Jiawei Guan, Qingling Wang, Huan Sun, Jianxun Qi, Jinghua Yan, Yan Chai, Zhongfu Wang, Chu-Xia Deng and George Gao
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## 1st Editorial Decision 6th Aug 2020

Dear Prof. Gao,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript, the referee reports from The EMBO Journal (attached again below) and your revision plan. All referees acknowledge that the findings are of interest. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, we feel all need to be addressed during a major revision (as also suggested by your revision plan). Importantly, as indicated by referee #3, the therapeutic consequences of the findings of the current manuscript have to be highlighted more strongly.

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/or in a detailed 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also our guide for figure preparation:
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We 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. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at:
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8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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9) Please add up to 5 key words to the title page.

10) Please note our new reference format:
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11) Please add a conflict of interest statement and a paragraph detailing the author contributions to the manuscript. Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends.

12) Please have your revised manuscript carefully proofread by a native speaker.

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.

Kind regards,

Achim

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Achim Breiling
Editor
EMBO Reports
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Referee #1:

In this manuscript, Liu et al. demonstrated that camrelizumab binds with PD-1 in a glycosylation-dependent manner, via analyzing the crystal structure of PD-1/ camrelizumab complex. The study provides structural insights into the detailed binding characteristics of camrelizumab and highlights the importance of glycosylation, especially N58, for camrelizumab function. They further showed that camrelizumab mainly utilizes the heavy chain to bind to PD-1 while the light chain of provides major steric hindrance for the competitive binding of PD-L1 to PD-1. Competitive antibody binding that blocks PD-L1 binding mainly involved the FG loop of PD-1.

Although authors reveal a glycosylation-dependent binding mode which is different from other approved PD-1 antibodies, including nivolumab or pembrolizumab. Most cell surface proteins are glycosylated and PD-1 glycosylation has been previously demonstrated. Glycan-dependent PD-1 folding and protein stability is expected as glycosylation is a general feature of protein quality control in the ER/Golgi pathway. Another PD-1 antibody with PD-1 N58 glycan-dependent binding has recently been functionally characterized (Sun et al., 2020). The structure of two other PD-1 antibodies (pembrolizumab and nivolumab) in complex with PD-1 has been solved (Horita et al., 2016; Tan et al., 2017). Therefore, it dampens novelty and conceptual advance of this manuscript. There are several major concerns listed below that need be to fully addressed to enhance scientific merits of the manuscript:

1. Based on the result showing the differences of camrelizumab in binding to PD-1 expressed in 293T cells, insect cells or E. coli (Fig.1), the authors concluded that the binding of camrelizumab with PD-1 was affected by glycosylation modification. More sufficient and direct evidence should be provided to support the conclusion. To better validate it, the authors could use PNGase to remove the glycans on PD-1 and then evaluate the binding affinity of camrelizumab with PD-1 expressed in 293T cells.
2. Authors relied on PD-1 expressed in 293T cells, while PD-1 is mainly expressed by T cells, where the protein may exhibit a different glycosylation profile. Primary T cells or a T cell line would be more appropriate for such study.
3. In Fig.2 and 3, the authors analyzed the overall structural binding characteristics of camrelizumab to PD-1 with other approved PD-1 antibodies, and showed that N-glycans on PD-1, especially N58, is contacted by camrelizumab. It will be of great interest if the authors would reveal the functional significance or effect of this glycosylation dependent binding mode, by comparing its binding affinity or efficacy with nivo or pembro, via additional in vitro or in vivo assays.
4. In Fig.4 and 5, the authors showed that N58 glycosylation is involved in camrelizuma binding, and is required for its function in blocking PD-1/PD-L1 interaction. The effect of N58 mutation on its cell surface expression should be taken into consideration. In addition, since the authors mentioned that all of the four glycosylated sites, including N58, are away from the binding surface with PD-L1, they should discuss the possibilities why and how binding with N58 glycosylation is crucial for camrelizumab to block PD-1/PD-L1 interaction.
5. In the discussion, the authors mentioned the concerns that camrelizumab might bind with conserved N-glycans presented on the other membrane proteins that share certain amino acid identities with PD-1. It will be better if the authors could use PD-1 knockout T cells or cancer cells to evaluate the binding specificity of camrelizumab.
6. PD-1 glycosylation has been shown in previous publications (Sun et al., 2020; Tan et al., 2017). A similar glycan-dependent PD-1 antibody has been functionally characterized (Sun et al., 2020). Glycan-dependent PD-1 folding and protein stability is expected as it is a general feature of protein quality control in the ER/Golgi compartments. Camrelizumab blocking efficiency of PD-L1 binding to

both WT and N58 mutant seems to be low, and difference between WT and N58A was only obvious when very high conc of the antibody was used (>100 ug/ml, Fig. 5). A comparison with other PD-1 antibodies for efficacy is needed, at least with pembro and nivo in vitro. Another glycan-dependent PD-1 antibody seems to perform better than these two in terms of potency (Sun et al., 2020).

Minor concerns:
1. There are some grammatical issues to be corrected. For example, in page 13, "Glycosylation dependent binding of camrelizumab may have profound influences to both PD-1/PD-L1 blocking efficiency and binding specificity", the word "to" should be replaced as "on"; in p5 para5, line 5 and 6 there are invalid references 17 and 18. It would be helpful to have a professional proofreading.
2. On page 12, the information of reference "15" was missing.
3. Standard nomenclature should be used to prevent confusion. For example. "N-acetylglucosamine (GlucNAc)" should be used in place of "N'acetylglucosamines (NAG)" (p8, line 2 from bottom).

References
Horita, S., Nomura, Y., Sato, Y., Shimamura, T., Iwata, S., and Nomura, N. (2016). High-resolution crystal structure of the therapeutic antibody pembrolizumab bound to the human PD-1. Sci. Rep. 6, 35297.
Sun, L., LI, C.-W., Chung, E.M., Yang, R., Kim, Y.-S., Park, A.H., Lai, Y.-J., Yang, Y., Wang, Y.-H., Liu, J., et al. (2020). Targeting glycosylated PD-1 induces potent anti-tumor immunity. Cancer Res. canres.3133.2019.
Tan, S., Zhang, H., Chai, Y., Song, H., Tong, Z., Wang, Q., Qi, J., Wong, G., Zhu, X., Liu, W.J., et al. (2017). An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat. Commun. 8, 14369.

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Referee #2:

The paper by Liu et al describes the interaction between the anti-PD1 antibody camrelizumab with PD-1, with a special emphasis of the binding dependent to N-glycosilation. This is an interesting study, in which the authors provide an example of a an anti-PD1 antibody that binds an antigenic site that is glycosilation-dependent. This in itself is not new, but this is the first anti-PD1 antibody that binds in such a way, and exhibits therapeutic activities. The authors also show that camrelizumab primarily binds PD-1 through the Vh region, and the Vl region probably provides sterical hindrance to PD-1 binding to PD-L1. Nevertheless, this is concluded mainly by comparison with the crystal structure of PD-L1/PD-1 complex.
The conclusions are supported by the data.

Minor concerns.

1. The authors say that "PD-1 is a highly glycosylated receptor expressed not only on T cells but also tumor cells. "
The expression of PD-1 in tumor cells is highly controversial. This reviewer has never detected it in cancer cells, when appropriate controls are used, and we have EXTENSIVELY tried to do so in human and mouse cancer cells. Indeed, PD-1 staining in tumor biopsies is restricted to T and B cells. Similarly, radioactively labelled PD-1 antibodies for in vivo visualization by PET does not significantly stain tumor tissue. Therefore, I suggest the authors to remove this, as it does not alter the paper. It is OK to mention it in the paper, but clarifying to the reader that not all researchers have observed this expression. Definitely, I would not state this in the abstract as the authors do.

2. There are some minor spelling mistakes throughout the manuscript (eucaryote instead of eukaryote, for example)
In the text there is no reference to Figure 1D, where it corresponds (page 6).

Major concerns.
This Reviewer would have liked the authors to show binding studies on other "conventional" anti-PD1 antibodies such as pembro or nivo to their N58-glycosilation-defective PD-1. I understand this will be dependent on whether the authors can get hold of these antibodies for these experiments. But I would strongly advise to do so, because otherwise the paper lacks some "in vitro activity" of the importance of N58 glycosilation on the efficacy of blockade over human T cells. I understand that these experiments could be quite complicated (for example, by CRISP-Cas9 elimination of endogenous PD-1 and re-expression of a N58 PD-1 mutant in Jurkat T cells).
If the authors can comply with the binding studies of conventional antibodies for just comparison, I would be happy to accept the paper.

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Referee #3:

Liu et al. report the crystal structure of PD-1 bound to a scFv version of camrelizumab and identified and characterized the role of the N-linked glycan on Asn58 in the high affinity binding interaction. Asn58 is the position of a SNP (rs1222512746: N58D) and as such, the involvement of the N-linked glycan is the binding to a therapeutic antibody is therefor of great interest. PD-1 is unregulated on antigen experienced T cells and one of the main targets of immune checkpoint blockade. Therapeutic antibodies that fail to block tumor expressed PD-L1 binding to PD-1 would not lead to tumor efficacy and the authors address the role of this glycan in the binding event.

The figures are clear and the manuscript is generally well -written and easy to follow. The manuscript builds up to a point where the authors finally suggest that camrelizumab binds the the N-linked glycan by itself and that could be the reason of its cross-reactivity with other receptors. This is an important point but the authors also need to better highlight the prevalence of this SNP. How commonly is it observed? How large could the patient population be in which the antibody fails to bind PD-1 with high affinity. The therapeutic consequences of the findings oof the current manuscript have to be highlighted more strongly.

Comments:

Are the SPR experiments done with IgG and only the crystallography with a refolded scFv dimer? Was there no monomeric scFv observed that could have been purified and used? A dimerize scFv is more a "wrongly associated" form of a single chain momoneric scFV and this should be discussed briefly. Even though it likely retains all of the structural features and binding properties.

Page 9 end of 1st paragraph. I would not say "binding dependency of PD-1 glycosylation..." but rather that N58 contributes to the high binding affinity of camrelizumab.

Page 9 last paragraph and Figure 4F. Since N-glycan binding cannot be measured reliably, one could argue that this experiment could be deleted form the manuscript. Here the authors need to make the point maybe in one sentences that this is important because it could form the basis of the non-specific binding that has described in the literature. The reader has to wait until the end to realize that this is an important experiment.

Page 10 first paragraph: While this reviewer appreciates that the glycan at N58 is involved in the binding affinity, the result of the staining experiments with wiltdytp or N58A -PD1 are surprising. The affinity of camrelizumab to N58A-PD1 is still much higher than that of PD-L1 and it is surprising that at a dose of 3.6mg/ml the antibody is unable to block PD-L1 binding. One reason could be that at that high concentration the IgG can only bind with one arm to PD-1 and loses its avidity effect and now the apparent faster off rate from PD1- N58 can be appreciated as PD-L1 can bind to free PD-1. Initially the blocking ability is similar for both wild type and N58A PD-1.
A better experiment would be to block with the Fab version of camrelizumab and use a different glycosylation deficient PD-1 as a negative control. Are the PD-1 cell surface expression levels similar?

Figure 5 is the most important experiment to support a role of this glycan in the efficacy of camrelizumab. At a dose of 28ug/ml however, no differences can be seen between wild type PD-1 and N58A PD-1. Is N58A the right mutation or is the alanine introducing hydrophobicity, which could result in a faster off-rate? How would N58S or N58Q behave?

The final sentence "Taken together, these results indicate that the blocking efficiency of camrelizumab to the interaction of N58 glycosylation deficient PD-1 and PD-L1 would be substantially attenuated. " is not correct unless a therapeutic camrelizumab dose is used.

Referee #1:

In this manuscript, Liu et al. demonstrated that camrelizumab binds with PD-1 in a glycosylation-dependent manner, via analyzing the crystal structure of PD-1/camrelizumab complex. The study provides structural insights into the detailed binding characteristics of camrelizumab and highlights the importance of glycosylation, especially N58, for camrelizumab function. They further showed that camrelizumab mainly utilizes the heavy chain to bind to PD-1 while the light chain of provides major steric hindrance for the competitive binding of PD-L1 to PD-1. Competitive antibody binding that blocks PD-L1 binding mainly involved the FG loop of PD-1.

Although authors reveal a glycosylation-dependent binding mode which is different from other approved PD-1 antibodies, including nivolumab or pembrolizumab. Most cell surface proteins are glycosylated and PD-1 glycosylation has been previously demonstrated. Glycan-dependent PD-1 folding and protein stability is expected as glycosylation is a general feature of protein quality control in the ER/Golgi pathway. Another PD-1 antibody with PD-1 N58 glycan-dependent binding has recently been functionally characterized (Sun et al., 2020). The structure of two other PD-1 antibodies (pembrolizumab and nivolumab) in complex with PD-1 has been solved (Horita et al., 2016; Tan et al., 2017). Therefore, it dampens novelty and conceptual advance of this manuscript. There are several major concerns listed below that need be to fully addressed to enhance scientific merits of the manuscript:

**Reply:**

**As the reviewer has commented above, PD-1 antibody with PD-1 N58 glycan-dependent binding has recently been functionally characterized (Sun et al., 2020). The structure of two other clinically approved PD-1 antibodies, pembrolizumab and nivolumab, in complex with PD-1 has been solved by our group and the others (Horita et al., 2016; Tan et al., 2017). This is the first report providing the structural evidence about a clinically approved anti-PD-1 antibody**

**directly targets PD-1 N58 glycan. Moreover, Finlay et al. have reported that camrelizumab showed low-affinity binding to other human receptors, such as vascular endothelial growth factor receptor 2 (VEGFR2), frizzled class receptor 5 and UL16 binding protein 2 (ULBP2). The most common side effects reported in clinical trials with camrelizymab included cutaneous reactive capillary endothelial proliferation, which may be correlated with these low affinity off-target bindings. The results presented in this study has provided a clue that the glycosylation binding dependency of camrelizumab may be responsible for these off-target interactions.**

1. Based on the result showing the differences of camrelizumab in binding to PD-1 expressed in 293T cells, insect cells or E. coli (Fig.1), the authors concluded that the binding of camrelizumab with PD-1 was affected by glycosylation modification. More sufficient and direct evidence should be provided to support the conclusion. To better validate it, the authors could use PNGase to remove the glycans on PD-1 and then evaluate the binding affinity of camrelizumab with PD-1 expressed in 293T cells.

**Reply:**

**We agreed with reviewer's suggestion and the binding between camrelizumab and PD-1 proteins expressed from 293T cells treated with PNGase was tested by SPR assay during this revision. The binding of camrelizumab to PNGase F treated PD-1 proteins was investigated, with PD-1 proteins without PNGase F treatment tested in parallel as a control. SDS-PAGE analysis revealed that the molecular weight of PD-1 protein was substantially reduced from ~35 kDa to ~20 kDa after treatment with PNGase F (revised Supplementary Fig. 5). The SPR analysis revealed that the binding affinity of camrelizumab to PD-1 proteins treated with PNGase has reduced to 105 nM, compared with the KD of 4.74 nM with PD-1 proteins without PNGase F treatment (revised Fig. 5G). We believe that both the structural evidence and N58A mutational analysis would also support the conclusions.**

2. Authors relied on PD-1 expressed in 293T cells, while PD-1 is mainly expressed by T cells, where the protein may exhibit a different glycosylation profile. Primary T cells or a T cell line would be more appropriate for such study.

**Reply:**

**We agreed that primary T cells or a T cell line would provide more valuable information for this study. However, precise control of the glycosylation in primary T cells or T cell line would be difficult for such analysis. Therefore, we mainly focused on the study of PD-1 glycosylation and its influences to camrelizumab interaction in the protein level or in 293T cells. Wild type or N58A mutated PD-1 expressed on 293T cell were tested for the blocking efficiency of camrelizumab to the interaction of PD-1 and PD-L1 with a new detection system, which is distinct from described in the previous manuscript version. The results revealed a substantially reduced blocking efficiency with N58 glycosylation site mutated PD-1, which is consistent with the protein level findings. The study of the influences of PD-1 glycosylation to camrelizumab interaction in primary T cell would provide more valuable information and should be carried out in the future. We also added this point in the discussion section as "As is observed in the present study, the PD-1/PD-L1 blocking efficiency of camrelizumab is substantially attenuated with N58A mutation, indicating the loss of T cell reactivation potency with camrelizumab. However, the functional influences of PD-1 glycosylation to camrelizumab administration in primary T cells still need further investigations."**

3. In Fig.2 and 3, the authors analyzed the overall structural binding characteristics of camrelizumab to PD-1 with other approved PD-1 antibodies, and showed that N-glycans on PD-1, especially N58, is contacted by camrelizumab. It will be of great interest if the authors would reveal the functional significance or effect of this glycosylation dependent binding mode, by comparing its binding affinity or efficacy with nivo or pembro, via additional in vitro or in vivo assays.

**Reply:**

**This is a good suggestion and we therefore carried out experiments to test the binding affinities of PD-1 and N58A with nivolumab or pembrolizumab by using SPR analysis. We found that the binding affinity ($K_D$) between wild type and N58A mutated PD-1 showed no substantial differences with nivolumab or pembrolizumab. These results were added as revised Figure 4H and I, and also described in the Result section. The previously reported complex structures of nivolumab or pembrolizumab with PD-1 also suggest that N58 is away from the binding area of these two mAbs. This indicates that N58-glycan didn't play a role in nivolumab or pembrolizumab binding to PD-1, and therefore N58A would not affect the blocking efficacy of these two mAbs.**

4. In Fig.4 and 5, the authors showed that N58 glycosylation is involved in camrelizuma binding, and is required for its function in blocking PD-1/PD-L1 interaction. The effect of N58 mutation on its cell surface expression should be taken into consideration. In addition, since the authors mentioned that all of the four glycosylated sites, including N58, are away from the binding surface with PD-L1, they should discuss the possibilities why and how binding with N58 glycosylation is crucial for camrelizumab to block PD-1/PD-L1 interaction.

**Reply: We agree with this suggestion and have added in the discussion section with this concern as "The binding affinity of camrelizumab to N58A mutated PD-1 has substantially reduced to 113 nM, which is ~24-fold lower than that to WT PD-1. The blocking efficiency of camrelizumab is not only dependent on the overlapping binding area of camrelizumab and PD-L1 on PD-1, but also the overwhelming binding affinity of camrelizumab to PD-1 than that of PD-L1. Therefore, the substantially reduced binding affinity of camrelizumab with N58A mutated PD-1 would result in attenuated blocking efficiency to PD-1/PD-L1 interaction, which is about 0.77–8.2 μM (Tan et al, 2016a)."**

5. In the discussion, the authors mentioned the concerns that camrelizumab might bind with conserved N-glycans presented on the other membrane proteins that share

certain amino acid identities with PD-1. It will be better if the authors could use PD-1 knockout T cells or cancer cells to evaluate the binding specificity of camrelizumab.

**Reply:**

**We have mentioned in the discussion that Finlay et al. have reported that camrelizumab showed low-affinity binding to other human receptors such as vascular endothelial growth factor receptor 2 (VEGFR2), frizzled class receptor 5 and UL16 binding protein 2 (ULBP2). The side effects reported in clinical trials with camrelizymab may be correlated with these low affinity off-target bindings. We agree that a full screening of the non-specific binding of camrelizumab is important for our understanding of the binding specificity of this mAb, but this may need extensive researches in the future. Besides, the previous findings and the data presented in the present study have provided useful information for our understanding of its non-specific binding.**

6. PD-1 glycosylation has been shown in previous publications (Sun et al., 2020; Tan et al., 2017). A similar glycan-dependent PD-1 antibody has been functionally characterized (Sun et al., 2020). Glycan-dependent PD-1 folding and protein stability is expected as it is a general feature of protein quality control in the ER/Golgi compartments. Camrelizumab blocking efficiency of PD-L1 binding to both WT and N58 mutant seems to be low, and difference between WT and N58A was only obvious when very high conc of the antibody was used (>100 ug/ml, Fig. 5). A comparison with other PD-1 antibodies for efficacy is needed, at least with pembro and nivo in vitro. Another glycan-dependent PD-1 antibody seems to perform better than these two in terms of potency (Sun et al., 2020).

**Reply:**

**It's really a good suggestion and we have changed to a distinct detection system to evaluate the blocking efficacy of the mAb to N58A mutation. In the previous study, we transfected full-length WT or N58A PD-1 genes with eGFP to 293 T cells and stained by PD-L1-mFc protein. It is hard to control the transfection efficiency to get a similar expression level based on GFP signal. In**

**the revised version, we have changed the detection system to stain the PD-L1 expressed on 293T cells with PD-1-mFc or N58A mutated PD-1-mFc proteins by FACS analysis. This enables that the ratio of camrelizumab and PD-1 proteins in the detection system could be accurate. The blocking efficiency of camrelizumab is determined with co-incubation of a serial of dilutions of camrelizumab and certain concentrations of WT or N58A mutated PD-1-mFc proteins, and the mixture of mAb and PD-1-mFc proteins were then used for staining of PD-L1 expressing 293T cells. The binding of PD-1-mFc to PD-L1 expressing 293T cells was completely abolished in the presence of 5 μg/mL of camrelizumab. On the other hand, the blocking of camrelizumab to the binding of N58A mutated PD-1 with PD-L1 is substantially attenuated that N58A mutated PD-1-mFc staining positive cells remained 34% even at a high concentration of 30 μg/mL, compared to 49% positivity with no camrelizumab. We also test the binding affinity of PD-1 and N58A with nivolumab or pembrolizumab and found that no substantial difference between PD-1 and N58A mutated PD-1 proteins in binding to nivolumab or pembrolizumab. Together with previously reported findings, all these evidence suggest that N58-glycan dose not play a role in nivolumab or pembrolizumab binding to PD-1, and would not affect the blocking efficacy to N58 glycosylation deficient PD-1.**

Minor concerns:

1. There are some grammatical issues to be corrected. For example, in page 13, "Glycosylation dependent binding of camrelizumab may have profound influences to both PD-1/PD-L1 blocking efficiency and binding specificity", the word "to" should be replaced as "on"; in p5 para5, line 5 and 6 there are invalid references 17 and 18. It would be helpful to have a professional proofreading.

**Reply: Thanks, the revised manuscript is further reedited by a native English speaker.**

2. On page 12, the information of reference "15" was missing.

**Reply: Thanks, we have modified accordingly.**

3. Standard nomenclature should be used to prevent confusion. For example. "N-acetylglucosamine (GlucNAc)" should be used in place of "N'acetylglucosamines (NAG)" (p8, line 2 from bottom).

**Reply:**

**Thanks, we have modified.**

References

Horita, S., Nomura, Y., Sato, Y., Shimamura, T., Iwata, S., and Nomura, N. (2016). High-resolution crystal structure of the therapeutic antibody pembrolizumab bound to the human PD-1. Sci. Rep. 6, 35297.

Sun, L., LI, C.-W., Chung, E.M., Yang, R., Kim, Y.-S., Park, A.H., Lai, Y.-J., Yang, Y., Wang, Y.-H., Liu, J., et al. (2020). Targeting glycosylated PD-1 induces potent anti-tumor immunity. Cancer Res. canres.3133.2019.

Tan, S., Zhang, H., Chai, Y., Song, H., Tong, Z., Wang, Q., Qi, J., Wong, G., Zhu, X., Liu, W.J., et al. (2017). An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat. Commun. 8, 14369.

Referee #2:

The paper by Liu et al describes the interaction between the anti-PD1 antibody camrelizumab with PD-1, with a special emphasis of the binding dependent to N-glycosilation. This is an interesting study, in which the authors provide an example of an anti-PD1 antibody that binds an antigenic site that is glycosilation-dependent. This in itself is not new, but this is the first anti-PD1 antibody that binds in such a way, and exhibits therapeutic activities. The authors also show that camrelizumab primarily binds PD-1 through the Vh region, and the Vl region probably provides sterical hindrance to PD-1 binding to PD-L1. Nevertheless, this is concluded mainly by comparison with the crystal structure of PD-L1/PD-1 complex.

The conclusions are supported by the data.

Minor concerns.

1. The authors say that "PD-1 is a highly glycosylated receptor expressed not only on T cells but also tumor cells. "

The expression of PD-1 in tumor cells is highly controversial. This reviewer has never detected it in cancer cells, when appropriate controls are used, and we have EXTENSIVELY tried to do so in human and mouse cancer cells. Indeed, PD-1 staining in tumor biopsies is restricted to T and B cells. Similarly, radioactively labelled PD-1 antibodies for in vivo visualization by PET does not significantly stain tumor tissue. Therefore, I suggest the authors to remove this, as it does not alter the paper. It is OK to mention it in the paper, but clarifying to the reader that not all researchers have observed this expression. Definitely, I would not state this in the abstract as the authors do.

**Reply:**

**Thanks, we agree with this suggestion and have removed accordingly in the abstract, but remained in the discussion section.**

2. There are some minor spelling mistakes throughout the manuscript (eucaryote instead of eukaryote, for example) In the text there is no reference to Figure 1D, where it corresponds (page 6).

**Reply:**

**We have reedited accordingly.**

Major concerns.

This Reviewer would have liked the authors to show binding studies on other "conventional" anti-PD1 antibodies such as pembro or nivo to their N58-glycosilation-defective PD-1. I understand this will be dependent on whether the authors can get hold of these antibodies for these experiments. But I would strongly advise to do so, because otherwise the paper lacks some "in vitro activity" of the

importance of N58 glycosilation on the efficacy of blockade over human T cells. I understand that these experiments could be quite complicated (for example, by CRISP-Cas9 elimination of endogenous PD-1 and re-expression of a N58 PD-1 mutant in Jurkat T cells).

If the authors can comply with the binding studies of conventional antibodies for just comparison, I would be happy to accept the paper.

**Reply:**

**As is also replied to Reviewer 1, this is really a good suggestion and we have done additional experiments to test the binding affinities of PD-1 and N58A with nivolumab or pembrolizumab by using SPR analysis. The results were presented in the revised Figure 4 and described in Results section. We found that the binding affinity ($K_D$) between wild type and N58A mutated PD-1 showed no substantial differences with nivolumab or pembrolizumab. The previously reported complex structures of nivolumab or pembrolizumab with PD-1 also suggest that N58 is away from the binding area of these two mAbs. This indicates that N58-glycan does not play a role in nivolumab or pembrolizumab binding to PD-1.**

Referee #3:

Liu et al. report the crystal structure of PD-1 bound to a scFv version of camrelizumab and identified and characterized the role of the N-linked glycan on Asn58 in the high affinity binding interaction. Asn58 is the position of a SNP (rs1222512746: N58D) and as such, the involvement of the N-linked glycan is the binding to a therapeutic antibody is therefor of great interest. PD-1 is unregulated on antigen experienced T cells and one of the main targets of immune checkpoint blockade. Therapeutic antibodies that fail to block tumor expressed PD-L1 binding to PD-1 would not lead to tumor efficacy and the authors address the role of this glycan in the binding event.

The figures are clear and the manuscript is generally well -written and easy to follow.

The manuscript builds up to a point where the authors finally suggest that camrelizumab binds the the N-linked glycan by itself and that could be the reason of its cross-reactivity with other receptors. This is an important point but the authors also need to better highlight the prevalence of this SNP. How commonly is it observed? How large could the patient population be in which the antibody fails to bind PD-1 with high affinity. The therapeutic consequences of the findings oof the current manuscript have to be highlighted more strongly.

**Reply:**

**We agree that the frequency of N58 variation is critical for the significance of this study. We checked the prevalence of N58 mutation of PD-1 in NCBI and found that a N58D polymorphism has been reported with an estimated frequency of 8 per million, as reported in TOPMed project reports. Therefore, the possibility of N58 variation correlated loss of high affinity binding to PD-1 do exist, though the frequency is low in the population. There is also possibility that altered glycosylation modification under certain conditions may also results in N58 glycosylation deficiency and further affects the binding of these mAbs that target glycosylation sites.**

Comments:

Are the SPR experiments done with IgG and only the crystallography with a refolded scFv dimer? Was there no monomeric scFv observed that could have been purified and used? A dimerize scFv is more a "wrongly associated" form of a single chain momoneric scFV and this should be discussed briefly. Even though it likely retains all of the structural features and binding properties.

**Reply:**

**As is described in Result section and in the Materials and Methods section, full length camrelizumab is used for SPR analysis with camrelizumab immobilized on the chip. For the scFv, we agree that the dimeric scFv is a “wrongly associated” form due to the short linker between VH and VL, but this**

**did not affect the binding properties with PD-1 and overall structural features. We have also discussed this in the Discussion section.**

Page 9 end of 1st paragraph. I would not say "binding dependency of PD-1 glycosylation..." but rather that N58 contributes to the high binding affinity of camrelizumab.

**Reply:**

**We agree that the glycosylation dependent binding may be not appropriate here. To give a more precise description, we have changed the first section of the Result to make sure that the varied binding affinity with PD-1 from different expression cells "may be" correlated with glycosylation. The description as this Reviewer has suggested has changed to "These results indicate that N58 glycosylation contributes to the high binding affinity of camrelizumab."**

Page 9 last paragraph and Figure 4F. Since N-glycan binding cannot be measured reliably, one could argue that this experiment could be deleted form the manuscript. Here the authors need to make the point maybe in one sentences that this is important because it could form the basis of the non-specific binding that has described in the literature. The reader has to wait until the end to realize that this is an important experiment.

**Reply:**

**We agree with this point that the quatitive binding to N-glycan may be not accurate and we could only detect a dose dependent binding to camrelizumab. We have added a comment about this point at the beginning of this section. Further studies should be carried out in the future to test whether the non-specific binding to other molecules, as is reported by Finlay et al, is also resulted in the binding to N-glycan in these molecules. The non-specific binding of the mAbs may depend on the contribution of binding of N-glycan compared with the overall interaction between PD-1 and mAbs, and the sequence similarity of amino acid residues. However, this may need extensive efforts to present a full**

**scenario of this answer and out of scope of this study.**

**We also conducted additional experiments to verify the reduced binding capacity of camrelizumab with PD-1 proteins treated with PNGase to remove the N-glycans. The binding between camrelizumab and PD-1 proteins expressed from 293T cells treated with PNGase was tested by SPR assay during this revision. The binding of camrelizumab to PNGase F treated PD-1 proteins was investigated, with PD-1 proteins without PNGase F treatment tested in parallel as a control. SDS-PAGE analysis revealed that the molecular weight of PD-1 protein was substantially reduced from ~35 kDa to ~20 kDa after treatment with PNGase F (revised Supplementary Fig. 5). The SPR analysis revealed that the binding affinity of camrelizumab to PD-1 proteins treated with PNGase has reduced to 105 nM, compared with the KD of 4.74 nM with PD-1 proteins without PNGase F treatment (revised Fig. 5G). We believe that both the structural evidence and N58A mutational analysis would also support the conclusions.**

Page 10 first paragraph: While this reviewer appreciates that the glycan at N58 is involved in the binding affinity, the result of the staining experiments with wiltdytp or N58A -PD1 are surprising. The affinity of camrelizumab to N58A-PD1 is still much higher than that of PD-L1 and it is surprising that at a dose of 3.6mg/ml the antibody is unable to block PD-L1 binding. One reason could be that at that high concentration the IgG can only bind with one arm to PD-1 and loses its avidity effect and now the apparent faster off rate from PD1- N58 can be appreciated as PD-L1 can bind to free PD-1. Initially the blocking ability is similar for both wild type and N58A PD-1.

A better experiment would be to block with the Fab version of camrelizumab and use a different glycosylation deficient PD-1 as a negative control. Are the PD-1 cell surface expression levels similar?

Figure 5 is the most important experiment to support a role of this glycan in the efficacy of camrelizumab. At a dose of 28ug/ml however, no differences can be seen between wild type PD-1 and N58A PD-1. Is N58A the right mutation or is the alanine

introducing hydrophobicity, which could result in a faster off-rate? How would N58S or N58Q behave?

**Reply:**

**As is also replied to Reviewer 1, we agree that the methods used here may be not appropriate for blocking analysis. Therefore, we have changed to another detection system to evaluate the blocking efficacy of the mAb to N58A mutation. In the previous methods, full-length WT or N58A PD-1 genes with eGFP were transfected to 293 T cells and stained by PD-L1-mFc protein. It is hard to control the transfection efficiency to get a similar expression level based on GFP signal. In the revised version, we have changed the detection system to detect the PD-L1 expressed on 293T cells with PD-1-mFc and N58A-mFc proteins by FACS analysis. The blocking efficiency of camrelizumab is determined with co-incubation of a serial of dilutions of camrelizumab and WT or N58A mutated PD-1-mFc proteins, and the mixture of mAb and PD-1-mFc proteins were then used for staining of PD-L1 expressing 293T cells. The binding of PD-1-mFc to PD-L1 expressing 293T cells was completely abolished in the presence of 5 μg/mL of camrelizumab. On the other hand, the blocking of camrelizumab to the binding of N58A mutated PD-1 and PD-L1 is substantially attenuated that N58A mutated PD-1-mFc staining positive cells remained 34% even at a high concentration of 30 μg/mL, compared to 49% positivity with no camrelizumab. We also test the binding affinity of PD-1 and N58A with nivolumab or pembrolizumab and found that no substantial difference between PD-1 and N58A mutated PD-1 proteins in binding to nivolumab or pembrolizumab. Together with previously reported findings, all these evidence suggest that N58-glycan didn't play a role in nivolumab or pembrolizumab binding to PD-1, and would not affect the blocking efficacy to N58 glycosylation deficient PD-1.**

The final sentence "Taken together, these results indicate that the blocking efficiency of camrelizumab to the interaction of N58 glycosylation deficient PD-1 and PD-L1 would be substantially attenuated. " is not correct unless a therapeutic camrelizumab

dose is used.

**Reply:**

**We agree with this suggestion and have changed "attenuated" to "reduced" in this sentence.**

## 1st Revision - Editorial Decision 11th Sep 2020

Dear Prof. Gao,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referee #2 (referee #1 from the submission to The EMBO Journal) has two remaining concerns, we ask you to address in a final revised manuscript.

After cross-commenting with referee #1 (referee #3 from the submission to The EMBO Journal) - see her/his comment below - we do not think it is necessary to evaluate the antibody in primary T cells or a T cell line. However, I agree with both referees that if you state that the antibody binds to the glycan itself on the surface of cells, solid data to demonstrate that is needed, and original point 5 of the referee needs to be addressed experimentally (maybe, as indicated by referee #1 in his/her cross-comment).

An alternative would be to take out the speculations about the binding to the glycan itself and to remove panel 4F and related discussions (as indicated in the cross-comments). In any case, please provide a point-by-point response addressing/ answering to the remaining points of referee #2 and the comment.

Moreover, I have these editorial requests:

- We would need a more comprehensive and simpler title. How about:
N-glycosylation of PD-1 promotes binding of Camrelizumab

- Please provide the abstract written in present tense.

- Please also make sure that in all the figure legends (including the abstract) the present tense is used.

- Please add a conflict of interest statement, and a paragraph detailing the author contributions to the manuscript (next to the acknowledgements).

- We would like to publish your manuscript as Report (as there are only 5 figures). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- In the figures, please use cells for the labeling (e.g. in Fig. 1 -> 293T cells, insect cells)

- Presently there are no call outs in the manuscript text for Fig. 4G and Appendix Figure S4. Please check and make sure that all panels in the figures have call outs.

- Please add a table of content (TOC) to the Appendix file and use the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables in the Appendix file according to this nomenclature.

- Please also note our new reference format. Please format your reference list accordingly: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Kind regards,

Achim Breiling
Editor
EMBO Reports

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Referee #1 (TEJ referee #3):

This manuscript is a transfer from EMBO J. I have previously reviewed this manuscript and consider this a revised version. The authors have adequately addressed all of my previous comments and concerns.

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Referee #2 (TEJ referee #1):

In this revised manuscript by Liu et al., authors addressed most of the comments. There are still concerns that were previously raised are not addressed at all. For example, the authors did not evaluate the antibody in the context of PD-1 expressed in T cells (point #2), as well as did not perform knockout PD-1 to validate the specificity of the antibody (point #5). These are important to confirm conclusion. The authors are encouraged to address these points to ensure scientific merit of the manuscript before it is acceptable for publication in EMBO Reports.

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Cross-comment of referee #1:

I think that the discussion about the precise nature of the glycans is blown out of proportion here. Since the antibody binds to both insect cell and mammalian cell (293T cell) N-linked glycans, it can only bind to the core structure that is conserved across the different N-linked glycan types, which is

demonstrated by the crystal structure. The core fucose is the only sugar that may or may not be present between different N-linked glycans. So precise structural variations in glycans that occur away from the core and thus the binding site of the antibody are not expected to have any role in binding.

So I think point 2 of the reviewer is not critical to address.

Point 5, I think, is a problem as the authors try to measure binding of the antibody to the naked glycan by itself. I overlooked Figure 4 panel F (the glycan binding SPR experiment). I don't believe this was a controlled experiment and since the authors don't know the concentration of the glycans used for the response it is difficult to judge that data. Therefore, the authors could eliminate panel F. Showing reduced binding in the absence of glycans is sufficient. If they are proposing the antibody binds the N-linked glycan, it would be important to show more complete data. A specific glycan immobilized on the chip, while passing the antibody over it would be the way to go. And then calculating the KD.

I would remove the speculation in the discussion that the antibody might bind to the glycan itself on the surface of cells unless they have solid data to demonstrate that. If they want to go this route I agree with reviewer #2 on point 5 that they need to show T cells. Maybe naive versus antigen experienced T cells can be used instead of PD-1 knock out, since they have different levels of PD-1 expression. That may be easier as one can observe the binding differences.

So my recommendation is to take out speculation about the binding to the glycan itself and remove panel 4F and related discussion.

----------------

Referee #1 (TEJ referee #3):

This manuscript is a transfer from EMBO J. I have previously reviewed this manuscript and consider this a revised version. The authors have adequately addressed all of my previous comments and concerns.

**Reply: Thank you for your comments.**

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In this revised manuscript by Liu et al., authors addressed most of the comments. There are still concerns that were previously raised are not addressed at all. For example, the authors did not evaluate the antibody in the context of PD-1 expressed in T cells (point #2), as well as did not perform knockout PD-1 to validate the specificity of the antibody (point #5). These are important to confirm conclusion. The authors are encouraged to address these points to ensure scientific merit of the manuscript before it is acceptable for publication in EMBO Reports.

**Reply: Thank you for your comments. We agreed that primary T cells or a T cell line would provide more valuable information for this study. However, precise control of the glycosylation in primary T cells or T cell line would be difficult for such analysis. As is also cross-commented by referee #1, the glycosylation variation in different cells may not be substantial to distinguish the binding differences as is observed in protein level in the manuscript. In protein based SPR analysis, no substantial difference of the binding affinity to camrelizumab was observed with PD-1 proteins expressed from insect cells or 293T cells.**

**Moreover, the structure of PD-1 and camrelizumab complex also showed that camrelizumab mainly binds to the core structure of N58 glycan. Therefore, the analysis in primary T cell or cell lines like Jurkat cells may not reflect the binding dependency of this mAb.**

**For point #5, we have to admit that the conclusion of direct binding to naked N-glycan is over-discussed. We don't have enough N-glycan from PD-1 to do the test and the SPR analysis could not be accurate that we could not determine the exact concentration of N-glycan. Therefore, we have removed this part in Figure 4F and related discussion in the manuscript.**

**We have mentioned in the discussion that Finlay et al. have reported that camrelizumab showed low-affinity binding to other human receptors such as vascular endothelial growth factor receptor 2 (VEGFR2), frizzled class receptor 5 and UL16 binding protein 2 (ULBP2). Considering that the core structure of N-glycan is similar in varied proteins, the binding specificity of the MAbs which showed glycosylation binding dependency may be lower than those that bind only to amino acids of PD-1. The results presented in this study has provided a clue that the glycosylation binding dependency of camrelizumab may be responsible for these off-target interactions.**

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Cross-comment of referee #1:

I think that the discussion about the precise nature of the glycans is blown out of proportion here. Since the antibody binds to both insect cell and mammalian cell (293T cell) N-linked glycans, it can only bind to the core structure that is conserved across the different N-linked glycan types, which is demonstrated by the crystal structure. The core fucose is the only sugar that may or may not be present between different N-linked glycans. So precise structural variations in glycans that occur away from the core and thus the binding site of the antibody are not expected to have any

role in binding.

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I would remove the speculation in the discussion that the antibody might bind to the glycan itself on the surface of cells unless they have solid data to demonstrate that. If they want to go this route I agree with reviewer #2 on point 5 that they need to show T cells. Maybe naive versus antigen experienced T cells can be used instead of PD-1 knock out, since they have different levels of PD-1 expression. That may be easier as one can observe the binding differences.

So my recommendation is to take out speculation about the binding to the glycan itself and remove panel 4F and related discussion.

**Reply: We agree with this suggestion and removed Figure 4F and related discussion in the revised manuscript.**

## 2nd Revision - Editorial Decision 22nd Sep 2020

Prof. George Gao
Institute of Microbiology,CAS
CAS Key Laboratory of Pathogenic Microbiology and Immunology
No. 1 Beichen West Road,Chaoyang District
Beijing, Beijing 100101
China

Dear Prof. Gao,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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## 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.**

## USEFUL LINKS FOR COMPLETING THIS FORM

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### 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? | NA |
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| 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. | NA |
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| 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. | NA |
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| 5. For every figure, are statistical tests justified as appropriate? | NA |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| Is there an estimate of variation within each group of data? | NA |

| | |
|---|---|
| Is the variance similar between the groups that are being statistically compared? | NA |

### C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | The antibody used for FACS is APC goat anti-mouse secondary IgG antibody (CAT: 405308; Biolegend, lot B30147) |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | HEK-293T cells are from ATCC and tested for mycoplasma contamination regulally in the lab. |

* 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. | NA |
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### 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

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| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | Data availability:<br>Atomic coordinates have been deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb) under accession code 7CU5. |
| 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). | NA |
| 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

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| 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 |