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Loss of VGLL4 suppresses tumor PD-L1 expression and immune evasion

Ailing Wu, Qingzhe Wu, Yujie Deng, Yuning Liu, Jinqiu Lu, Liansheng Liu, Xiaoling Li, Cheng Liao, Bin Zhao, Hai Song

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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 3rd May 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments the referees find the topic and analysis interesting, but also that further revisions are needed to consider publication here. Should you be able to address the raised concerns in full then we would be interested in considering a revised version.

I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the major concerns raised at this stage.

REFEREE REPORTS:

Referee #1:

This manuscript by Wu et al. studies the largely unknown roles of Vgll4 in anti-tumor immunity. Overall the authors present a good set of data covering a lot of ground. Most data sets are very clearly presented and are convincing. However, I think that some additional experiments are needed to verify the model proposed in Figure 7. In particular the role(s) of YAP as proposed in this model should be addressed by some additional experiments; otherwise the emphasis regarding the Hippo pathway will need to be corrected for the entire manuscript.

Main points
1) Emphasis on the Hippo pathway in the abstract
In the abstract the authors state that " ... highlights a central role of VGLL4 and Hippo pathway in the regulation of tumor immunity." Considering that the Hippo pathway is clearly more than YAP alone and that the majority of the experiments presented in this manuscript are not focused on YAP, I do not think that it is a good idea to put Vgll4 and the Hippo pathway on the same level of

emphasis in the abstract. In other words, I highly recommend to correct this over-emphasis of the Hippo pathway in the abstract; otherwise, I would be very tempted to ask the authors for additional experiments regarding other Hippo components such as MST1/2, LATS1/2 and others.

2) Figure 1
The data presented in Figure 1 are good and represent well controlled experiments. I would only have one point regarding Figure 1: Could the authors please verify that Vgll4 is still knocked down in tumours developed by shVgll4 cells in nude mice. I am raising this point because it could be that Vgll4 expression is restored in immune compromised animals, which could be an alternative explanation for the observed results.

3) Figure 2
Like Figure 1, also a set of good data, but I think that additional experiments should be added to complete this figure. The main text should also be slightly adjusted as outlined below.
3a) On page 6 the authors state that " ... indicating that VGLL4 regulates the transcription of PD-L1." I strongly suggest to re-phrase this sentence by rather stating that Vgll4 expression levels correlate with PD-L1 expression levels.
3b) On page 7 the authors state that "These data suggest that loss of VGLL4 suppresses PD-L1 expression in tumor cells, leading to the establishment of antitumor immunity." In this regard, I would like to see one additional experiment to solidify this conclusion even further. More specifically, I am suggesting the following experimental expansion:
Include one rescue experiment to complete Figure 2. For example, study the consequence of exogenous expression of Vgll4 in KO cells as described in Figure 2J. Is the expression of endogenousPD-L1 restored upon re-expression of Vgll4?
3c) On page 7 the authors state that "...two VGLL4 mutants, ΔTDUs and HF4A (H212A/F213A/H240A/F241A) (Jiao et al, 2017), both of which have been shown to lose the ability to interact with the YAP-binding domain of TEADs." I think that these Vgll4 mutants would be ideal candidates to be tested in parallel to wild-type Vgll4 as proposed in point 3b above. By functionally testing these mutants in KO cells with regard to their ability to rescue PD-L1 expression the importance of the YAP/TEAD interaction can be addressed.

4) Figure 3
The main message of this figure is clearly stated by the authors as "VGLL4 interacts with IRF2BP2, and that TDU domains in VGLL4 are not required for the interaction with IRF2BP2 and the regulation of PD-L1 expression." Overall, this figure presents convincing data sets. I just wondering about the testing of Vgll4 mutants as tested in this figure in the context described in point 3c above.

5) Figure 4
The main message of this figure is also clearly defined by the authors as "...suggest that VGLL4 protect polyubiquitination and proteasome-dependent degradation of IRF2BP2." The presented data are overall satisfactory in this Figure. However, I think that a quantification of three independent experiments should be included for Figure 4G to fully solidify this important data set.

6) Figure 5
Also for this figure the main message is clearly defined by the authors in the main text as "... IFNγ stimulation triggers the release of IRF2 from PD-L1 promoter and dynamic interaction between IRF2 and IRF2BP2." The presented experiments are overall satisfactory in this Figure, but I nevertheless still think that the following 2 points should be considered to improve this figure further:
6a) Quantification of the data shown in Figure 5E (ideally from three independent experiments)
6b) Quantification of data shown in Figure EV3

7) Figure 6 and EV4
The authors state in the main text the following main message: " .... activation of YAP inhibits IFNγ-inducible PD-L1 expression through its target genes." While this figure also presents a good amount of good data, I think that additional points need to be considered:
7a) Figure 6E and 6F - What happens when wild-type YAP is expressed? The two studied YAP versions do not exist in nature, so the testing of a wild-type version is rather important.
7b) The result using the S94A mutant indicates that the inhibition is in part independent of the YAP/TEAD interaction. In this regard, it would be very good to test this in the context of point 3c

outlined above (basically Vgll4 mutants that cannot interfere with YAP/TEAD should be tested regarding PD-L1 expression).
7c) Understand in more detail the consequences of IFNy treatment on the Hippo pathway- What happens to endogenous YAP upon IFNy treatment? More specifically, what happens to the phosphorylation / activity / localisation status of YAP? In other words, does IFNy "turn off" endogenous YAP, hence allowing the induction of PD-L1 expression? (without such an experiment on endogenous YAP, the current links between YAP and IFNy are rather weak and mainly based on YAP overexpression, which could be quite misleading)

8) Figure 7/EV Table 1
The main message of this figure is also clearly defined by the authors as "Human epidemiological data strongly suggest that the expression levels of VGLL4 are clinically relevant and that lower expression of VGLL4 correlates with better patient outcome." Given the emphasis of the Hippo pathway (aka YAP) in this manuscript and the final model shown in Figure 7, I have been very tempted to ask for the following additional experiments:
8a) In the context of Figure 7A: What is the YAP expression / localisation in these tumour samples? Is there correlation of Vgll4 and YAP?
8b) The model shown in Figure 7D puts a clear emphasis on YAP in the context of PD-L1. More specifically, based on this model YAP5SA overexpression should to be sufficient suppress tumour immune evasion. In other words the overexpression of YAP (a YAP version that cannot be touched by Vgll4; or maybe overexpression of wild-type or 5SA is also sufficient?) would be expected to decrease tumour growth in for example LLC cells as tested in Figure 1C. Is this actually the case?

Minor points
A) Expand the discussion to speculate also on additional points introduced earlier
In the introduction section the authors state that: "Many studies reported that the dysregulation of several oncogenic or tumor-suppressive pathways constitutively activates the expression of PD-L1, suggesting that this is a general mechanism of tumorigenesis. PTEN deletions, PI3K/AKT mutations (Lastwika et al, 2016; Parsa et al, 2007), EGFR mutations (Akbay et al, 2013), MYC overexpression(Casey et al, 2016), CDK5 disruption (Dorand et al, 2016) and YAP/TAZ activation (Feng et al, 2017; Janse van Rensburg et al, 2018; Kim et al, 2018; Lee et al, 2017; Miao et al, 2017) represent a rapidly growing list of genetic mechanisms of constitutive PD-L1 expression.". I can fully understand that the authors focus in their manuscript on the link between Vgll4 and YAP; however, I think that it could be interesting to speculate also in how far the manipulation of Vgll4 maybe also affects other cancer pathways (as mentioned here in the introduction section) in the context of PD-L1 expression.

B) Improve Figure 2G
Please improve the presentation of Figure 2G, so that it is easier to be understood.

C) Define YAP cDNAs in the M&M section
The authors need to very clearly define which YAP cDNAs (isoforms) were used in their study.

Referee #2:

The study by Wu et al describes a highly detailed series of analyses to show that VGLL4 plays a critical role in T cell anti-tumor activity. Figure 1 and EVF1 and 2 clearly show that this phenomenon is present in immunocompetent mice and absent in nude mice in tumors arising from 2 cell lines that highly express VGLL4 (LLC and MB49). They go on to show that the cells themselves grow at identical rates regardless of the presence/absence of VGLL4 in vitro, that in vivo depletion of CD8/CD4 T cells restores the VGLL4 knockout cells' tumor growth in immunocompetent mice, and that PD-L1 blockade eliminates tumor growth in wild type tumors.

However, the subsequent experiments shown by the authors utilize a large number of different cell lines inconsistently to demonstrate various aspects of VGLL4's ultimate interaction with PD-L1, IRF2BP2, and YAP. This broadly represents the greatest weakness of the paper, as this potentially limits the translatability of the results (and should certainly be acknowledged). In figure 2, MB49 (one of the cell lines used for the mouse work) is shown to have modestly reduced PD-L1 expression in response to VGLL4 knockdown, as well as reduced B7-H4, which is another negative

regulator of T cell activity. The remainder of the figure demonstrates in a variety of other cell lines not used in animal work (mainly A549) that VGLL4 knockdown downregulates PD-L1 expression (seemingly moreso than in MG549, LCC is not assessed in this way except for augmentation of PD-L1 expression in the final panel), and that this seems to happen despite seemingly intact interferon signaling via STAT1 due to PD-L1 promoter-specific suppression. The authors go on to demonstrate VGLL4's interaction with IRF2BP2 via inhibiton of ubiquination and subsequent proteasome degradation (using the A549 and HEK293T lines). IRF2BP2 knockout is shown to have a modest effect on PD-L1 expression via IF2 interaction with the PD-L1 promoter in the setting of IFN stimulation in A549 cells, and YAP/miR-130a is shown to inhibit IFN induced PD-L1 and IRF1 expression in A549 cells. Finally, the authors examine a clinical database to correlate VGLL4 with survival in lung cancer patients, and show its expression in lung cancer tissue and its association with IRF3BP2 expression. The overall thesis of the authors is certainly novel, and the level of detail in which the molecular mechanism is studied is impressive. However, there are several questions/concerns which arose during my review of the manuscript:

Major points:
- The authors' initial animal work is thorough and compelling. However, the subsequent experiments are largely conducted on the A549 cell line (and several others). Why were these cell lines used instead of the initial LCC and MB49 lines? Was any attempt made to grow A549 in mice to recapitulate these results? Or were the various downstream experiments ever tried with the LCC/MB49 lines? While the degree of work performed is exhaustive and recapitulating many experiments with new cell lines may not be feasible, this weakness in extrapolation should be heavily addressed in the discussion section.

- The downregulation of PD-L1 in response to VGLL4 knockdown was ultimately rather modest in MB49. Did the authors study its downregulation in LCC? Additionally, other T cell inhibitory factors (B7-H4) are also downregulated in response to VGLL4 knockdown. Did the authors ever study the responsiveness of these cells to inferferon-mediated cell killing? It would seem that the degree of PD-L1 downregulation might only be one contributor to the data seen in Figure 1/EV1/EV2, and it may be a more holistic reversal of T cell exclusion.

-Figure 1K shows only 1 field of each stain in each type of animal. Having a more quantitative measure of increased CD3/CD8/CD45 cells would be more convincing, such as cells/high-powered field (take average of multiple fields per tumor per animal). This would enable statistical comparison of the different amounts of marker-positive cells.

-The methods used to generate Figure 7 are not described in the materials and methods. There is no description of what the authors used for high/low cutoffs of VGLL4 expression. Additionally, if the authors attempted a hazard ratio calculation (of risk of death) it might be more compelling than an arbitrary cutoff value and associated survival curves (although both could certainly be reported, particularly if the hazard ratio is consistent with the trend reported).

-The methods used to generate the IHC data in figure 7 is not described in materials and methods. Also, how did the authors quantify the degree of expression? Positive cells/high powered field would be preferable, but IHA score is another option (it is unclear how the authors scored these samples, as well as all methods for the slides' generation/staining).

Minor points:
-For figure EV2, the data would benefit from also showing the tumor volume curves in addition to the comparison of the tumor weight.

-For the flow cytometry data in Figure 2, representative gating would be helpful (this can be a supplemental figure).

-Figure 5A - the significance between various bars in the graph should be more clearly delineated with connecting lines as in 5B and 5C.

-In general, the figure legends are very brief. Unless forbidden for editorial reasons, more descriptive legends and labeling of the various cell lines used for the various experiments would greatly help the readers.

-There are multiple minor instances where the English syntax could be improved by a copy editor.

Referee #3:

The manuscript titled "Loss of VGLL4 suppresses tumor PD-L1 expression and immune evasion" by Wu et. al. show that VGLL4 deficiency in tumor cells reduces PD-L1 expression. The manuscript provides an experimental evidence for VGLL4's novel role in anti-tumor immunity. Loss of VGLL4 reduces PD-L1 expression in lung cancer cells. VGLL4 regulates PD-L1 expression by modulating IRF2/IRF2BP2 complex. VGLL4 binds IRF2BP2 and stabilizes IRF2BP2 from proteasome-mediated protein degradation. In addition, the authors show that YAP activation inhibits VGLL4 and IRF1 expression through miR-130a and supresses IFNγ-inducible PD-L1 expression. Present finding on the VGLL4 as an important regulator of PD-L1 expression is interesting and novel. There are some concerns for this manuscript.

Major
1. How stabilization of IRFBP2 by Vgll4 sustained PD-L1 was not really addressed. Since the author speculated that IRF2 may have increased binding to PD-L1 promoter, then knockdown of IRF2 together with Vgll4 silence should restore L1 expression and tumor growth?
2. Over expression of Vgll4 in low expression cells should be performed and it can promote PD-L1 expression and tumor growth in immune competent but not deficient mice.
3. Since Vgll1-3 did not interact with IRFBP2, their silence should not affect PD-L1 expression and this should be done and shown.
4. How YAP-suppressed IRF1 and PD-L1 expression is linked to Vgll4? Are we talking about one integrated regulation or two independent regulations? More mechanistic details are needed in addition to simply drawing a model.
5. YAP inhibits IFNγ-inducible PD-L1 expression through miRNA-130a-mediated suppression of VGLL4 and IRF1 expression? It is important to assess the relevant contributions of Vgll4 vs IRF1?
6. IRF1 over-expression should restore the suppression of PD-L1 imposed by Vgll4 silence or YAP overexpression as well as Tumor growth?
7. A rescue with VGLL4 that is mutant to siRNA effects is required to show the restoration of PD-L1 levels in A549, and subsequent tumorigenic effects in syngeneic mice models. Also, the PDL1 levels in A549 does not seem to correlate with the level of knockdown of VGLL4 si-1 and 2 in Fig 2D. Moreover, the rationale for using A549 cells is unclear to me, when LLC cell lines have the most VGLL4 expression amongst lung cancer cells, as shown in Fig 1A.
8. The authors interpret that TDU domains are not required for IRF2BP2-VGLL4 interactions; however, at later stages (Fig 6), their data imply that binding of YAP-TEAD controls PDL1 expression under IFN response. In that case, what is the status of VGLL4-TEAD versus TEAD-YAP complexes in these cell types with or without IFN induction? In other words, which partnering complex is more prevalent upon IFN induction?
9. A knockdown of TEAD in the VGLL4 kd A549 cells is recommended to denote its effects on IRF2BP2 and PDL1, respectively.
10. What is the status of YAP expression and their targets, and miR130a, in A549 cells that were depleted of VGLL4? The expression of YAP and its targets also needs to be studied in VGLL4 deficient tumors that were rescued by mouse-PDL1.
11. Despite robust effects on VGLL4, I do not see a corresponding significant effect of miR130-a mimic or sponge in down- and up-regulating PDL1 expression (Fig 6a-b). For instance, in panel b, the levels of VGLL4 are strongly up by 12h post IFN induction, whereas PDL1 levels are not. This gives the reader an impression that miR130a route may not be the major regulator of PDL1 even though it may regulate VGLL4.
12. Why was the expression pattern of PDL1 not included in the lung cancer specimens in Fig 7A? Also, what is the YAP expression profile in these lung cancer specimens that have high VGLL4-IRF2BP2 expression?

We would like to thank the reviewers for their careful reading and constructive criticism of our manuscript. We have made the following changes and added experiments per their recommendations, which are addressed point-by-point below:

Referee #1:

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Main points
1) Emphasis on the Hippo pathway in the abstract
In the abstract the authors state that " ... highlights a central role of VGLL4 and Hippo pathway in the regulation of tumor immunity." Considering that the Hippo pathway is clearly more than YAP alone and that the majority of the experiments presented in this manuscript are not focused on YAP, I do not think that it is a good idea to put Vgll4 and the Hippo pathway on the same level of emphasis in the abstract. In other words, I highly recommend to correct this over-emphasis of the Hippo pathway in the abstract; otherwise, I would be very tempted to ask the authors for additional experiments regarding other Hippo components such as MST1/2, LATS1/2 and others.

(Response) We appreciate the reviewer's point that Hippo pathway is a broader concept and we did not explore the role of other Hippo components in the regulation of PD-L1 expression. We have changed Hippo pathway to YAP in the abstract on page 2, line 16.

2) Figure 1
The data presented in Figure 1 are good and represent well controlled experiments. I would only have one point regarding Figure 1: Could the authors please verify that Vgll4 is still knocked down in tumours developed by shVgll4 cells in nude mice. I am raising this point because it could be that Vgll4 expression is restored in immune compromised animals, which could be an alternative explanation for the observed results.
(Response) As reviewer's suggestion, we examined the VGLL4 protein levels in the tumor lysates from nude mice by western blot and found that VGLL4 protein levels were still low in the VGLL4 depleted LLC and MB49 tumors (revised Fig. EV1D and EV1F).

3) Figure 2

Like Figure 1, also a set of good data, but I think that additional experiments should be added to complete this figure. The main text should also be slightly adjusted as outlined below.

3a) *On page 6 the authors state that " ... indicating that VGLL4 regulates the transcription of PD-L1." I strongly suggest to re-phrase this sentence by rather stating that Vgll4 expression levels correlate with PD-L1 expression levels.*

(Response) We greatly appreciate the reviewer's point that we need to correct the word used in the conclusion. We have changed the sentence to a more appropriate conclusion as the reviewer's suggestion on page 7, line 6.

3b) *On page 7 the authors state that "These data suggest that loss of VGLL4 suppresses PD-L1 expression in tumor cells, leading to the establishment of antitumor immunity." In this regard, I would like to see one additional experiment to solidify this conclusion even further. More specifically, I am suggesting the following experimental expansion:*

Include one rescue experiment to complete Figure 2. For example, study the consequence of exogenous expression of Vgll4 in KO cells as described in Figure 2J. Is the expression of endogenousPD-L1 restored upon re-expression of Vgll4?

(Response) As reviewer's suggestion, we performed a rescue experiment. Because WT-VGLL4 greatly suppresses YAP activity and inhibits cancer cell proliferation, we used VGLL4-HF4A to rescue PD-L1 expression in VGLL4 knockdown cells. We found that VGLL4-HF4A efficiently restored the PD-L1 expression in VGLL4 depleted A549 cells (revised Fig. 3J).

3c) *On page 7 the authors state that "...two VGLL4 mutants, ΔTDUs and HF4A (H212A/F213A/H240A/F241A) (Jiao et al, 2017), both of which have been shown to lose the ability to interact with the YAP-binding domain of TEADs." I think that these Vgll4 mutants would be ideal candidates to be tested in parallel to wild-type Vgll4 as proposed in point 3b above. By functionally testing these mutants in KO cells with regard to their ability to rescue PD-L1 expression the importance of the YAP/TEAD interaction can be addressed.*

(Response) To address the reviewer's point, we performed several experiments to test VGLL4-HF4A function. Because WT-VGLL4 overexpression significant inhibits cell growth in several cell types including lung cancer cells, we did not use WT-VGLL4. We found that 1) VGLL4-HF4A efficiently rescued PD-L1 expression in VGLL4 depleted A549 cells (revised Fig. 3J). 2) VGLL4-HF4A alleviated T cell-mediated cancer cell killing in vitro (revised Fig.3L). 3) VGLL4-HF4A restored the tumor growth of Vgll4 knockdown LLC cells in murine syngeneic model (revised Fig. 3K). 4) VGLL4-HF4A B16F10 tumors grew faster in murine syngeneic model (Fig. EV3B). These data suggest that VGLL4 regulates PD-L1 expression and anti-cancer immunity independent of TEADs.

4) Figure 3

The main message of this figure is clearly stated by the authors as "VGLL4 interacts

with IRF2BP2, and that TDU domains in VGLL4 are not required for the interaction with IRF2BP2 and the regulation of PD-L1 expression." Overall, this figure presents convincing data sets. I just wondering about the testing of Vgll4 mutants as tested in this figure in the context described in point 3c above.
(Response) We appreciate the reviewer's suggestion to further clarify the relationship among VGLL4, TEADs and IRF2BP2. We performed a new experiment in the revised Fig. EV4A and EV4B. We found that there were increased IRF2BP2 proteins in the TEADs-depleted A549 cells. We reasoned that due to the depletion of TEADs, more VGLL4 proteins were released from TEADs/VGLL4 complex and bound to IRF2BP2 proteins to stabilize IRF2BP2 proteins. However, we did not observe the increased PD-L1 expression, which may be due to the decreased TEADs/YAP transcriptional activity in TEADs knockdown cells.

5) Figure 4
The main message of this figure is also clearly defined by the authors as "...suggest that VGLL4 protect polyubiquitination and proteasome-dependent degradation of IRF2BP2." The presented data are overall satisfactory in this Figure. However, I think that a quantification of three independent experiments should be included for Figure 4G to fully solidify this important data set.
(Response) As reviewer's suggestion, we have quantified the IRF2BP2 protein levels in revised Fig. 4G.

6) Figure 5
Also for this figure the main message is clearly defined by the authors in the main text as "... IFNγ stimulation triggers the release of IRF2 from PD-L1 promoter and dynamic interaction between IRF2 and IRF2BP2." The presented experiments are overall satisfactory in this Figure, but I nevertheless still think that the following 2 points should be considered to improve this figure further:

6a) *Quantification of the data shown in Figure 5E (ideally from three independent experiments)*
(Response) We have quantified the relative PD-L1 levels in the revised Fig. 5G as the reviewer's suggestion. We also included an additional IRF2BP2-KO A549 cells in the revised Fig. EV5B.

6b) *Quantification of data shown in Figure EV3*
(Response) We have quantified the relative VGLL4 protein levels in the revised Fig. EV5C.

7) Figure 6 and EV4
The authors state in the main text the following main message: " .... activation of YAP inhibits IFNγ-inducible PD-L1 expression through its target genes." While this figure also presents a good amount of good data, I think that additional points need to be considered:

7a) *Figure 6E and 6F - What happens when wild-type YAP is expressed? The two studied YAP versions do not exist in nature, so the testing of a wild-type version is rather important.*
(Response) We agree with the review that the activity of WT-YAP was missing in the original manuscript. We have included the data, which showed that WT-YAP also suppressed IFNγ-inducible PD-L1 expression in the revised Fig. EV6C.

7b) *The result using the S94A mutant indicates that the inhibition is in part independent of the YAP/TEAD interaction. In this regard, it would be very good to test this in the context of point 3c outlined above (basically Vgll4 mutants that cannot interfere with YAP/TEAD should be tested regarding PD-L1 expression).*
(Response) In our model, inhibition of IFNγ-inducible PD-L1 by YAP mainly through miR-130a. We performed an additional experiment in the revised Fig. 6L. We generated a miR130a KO cell line, which is more clean than miR-130a sponge. We found that deletion of miR-130a cannot completely release the suppression by YAP5SA, which indicate that the inhibition is in part mediated by the YAP/TEAD transcription. Interestingly, one study reported that IFNγ-inducible expression of PD-L1 was dependent on NF-κB (Gowrishankar et al, 2015). In our study, we showed that YAP also suppressed TNFα-inducible PD-L1 expression through inhibiting NF-κB signaling, which did not require the transcriptional activity of YAP (YAP-S94A). Alternatively, YAP may regulate IFNγ-inducible expression of PD-L1 through NF-κB signaling. The detailed mechanism of the crosstalk between Hippo-YAP and TNFα-NF-κB will be published elsewhere. We have toned down the role of miR130a in regulating the suppression of PD-L1 expression by YAP and discuss this in the discussion on page 17.

7c) *Understand in more detail the consequences of IFNy treatment on the Hippo pathway- What happens to endogenous YAP upon IFNy treatment? More specifically, what happens to the phosphorylation / activity / localisation status of YAP? In other words, does IFNy "turn off" endogenous YAP, hence allowing the induction of PD-L1 expression? (without such an experiment on endogenous YAP, the current links between YAP and IFNy are rather weak and mainly based on YAP overexpression, which could be quite misleading)*
(Response) We appreciate the reviewer's suggestion to clarify whether IFNγ treatment affects YAP activity. We have performed the experiments as reviewer's suggestion in the revised Fig. EV6E-EV6G. We found that INFγ treatment didn't affect the endogenous YAP phosphorylation and localization.

8) Figure 7/EV Table 1
The main message of this figure is also clearly defined by the authors as "Human epidemiological data strongly suggest that the expression levels of VGLL4 are clinically relevant and that lower expression of VGLL4 correlates with better patient outcome." Given the emphasis of the Hippo pathway (aka YAP) in this manuscript and the final model shown in Figure 7, I have been very tempted to ask for the

following additional experiments:
8a) *In the context of Figure 7A: What is the YAP expression / localisation in these tumour samples? Is there correlation of Vgll4 and YAP?*
(Response) We have performed the analysis as reviewer's suggestion. However, we didn't observe a significant correlation of VGLLL4 and YAP expression in the revised Fig. EV7A, which indicates that a more complex regulation in the clinical samples.

8b) *The model shown in Figure 7D puts a clear emphasis on YAP in the context of PD-L1. More specifically, based on this model YAP5SA overexpression should to be sufficient suppress tumour immune evasion. In other words the overexpression of YAP (a YAP version that cannot be touched by Vgll4; or maybe overexpression of wild-type or 5SA is also sufficient?) would be expected to decrease tumour growth in for example LLC cells as tested in Figure 1C. Is this actually the case?*

(Response) We have performed the experiment as reviewer's suggestion in the revised Fig. 6G. Overexpression of WT-YAP decreased LLC tumor growth in murine syngeneic tumor model, not in nude mice, which is consistent with the previous study about the role of Hippo and YAP in anti-cancer immunity (Moroishi et al, 2016). Due to the profound effect of YAP activation, we did not explore the detailed mechanism of this phenotype in the current study. We discussed this on page 16 and 17.

Minor points

A) Expand the discussion to speculate also on additional points introduced earlier
In the introduction section the authors state that: "Many studies reported that the dysregulation of several oncogenic or tumor-suppressive pathways constitutively activates the expression of PD-L1, suggesting that this is a general mechanism of tumorigenesis. PTEN deletions, PI3K/AKT mutations (Lastwika et al, 2016; Parsa et al, 2007), EGFR mutations (Akbay et al, 2013), MYC overexpression(Casey et al, 2016), CDK5 disruption (Dorand et al, 2016) and YAP/TAZ activation (Feng et al, 2017; Janse van Rensburg et al, 2018; Kim et al, 2018; Lee et al, 2017; Miao et al, 2017) represent a rapidly growing list of genetic mechanisms of constitutive PD-L1 expression.". I can fully understand that the authors focus in their manuscript on the link between Vgll4 and YAP; however, I think that it could be interesting to speculate also in how far the manipulation of Vgll4 maybe also affects other cancer pathways (as mentioned here in the introduction section) in the context of PD-L1 expression.
(Response) As reviewer's suggestion, we have examined several pathways in VGLL4 knockdown cells in revised Fig. EV2E. Interestingly, we found that PTEN levels were reduced in VGLL4 knockdown A549 and MB49 cells. We did not further address the significance of this finding in the revised manuscript.

B) Improve Figure 2G
Please improve the presentation of Figure 2G, so that it is easier to be understood.

(Response) We have rewritten the description for original Figure 2G.

C) Define YAP cDNAs in the M&M section
(Response) YAP cDNAs is from Addgene. We have included the information in M&M on page 19, line 4.

The authors need to very clearly define which YAP cDNAs (isoforms) were used in their study.
(Response) It is YAP2 from Addgene. We have included the information in M&M on page 19, line 4.

Referee #2:

The study by Wu et al describes a highly detailed series of analyses to show that VGLL4 plays a critical role in T cell anti-tumor activity. Figure 1 and EVF1 and 2 clearly show that this phenomenon is present in immunocompetent mice and absent in nude mice in tumors arising from 2 cell lines that highly express VGLL4 (LLC and MB49). They go on to show that the cells themselves grow at identical rates regardless of the presence/absence of VGLL4 in vitro, that in vivo depletion of CD8/CD4 T cells restores the VGLL4 knockout cells' tumor growth in immunocompetent mice, and that PD-L1 blockade eliminates tumor growth in wild type tumors.

However, the subsequent experiments shown by the authors utilize a large number of different cell lines inconsistently to demonstrate various aspects of VGLL4's ultimate interaction with PD-L1, IRF2BP2, and YAP. This broadly represents the greatest weakness of the paper, as this potentially limits the translatability of the results (and should certainly be acknowledged). In figure 2, MB49 (one of the cell lines used for the mouse work) is shown to have modestly reduced PD-L1 expression in response to VGLL4 knockdown, as well as reduced B7-H4, which is another negative regulator of T cell activity. The remainder of the figure demonstrates in a variety of other cell lines not used in animal work (mainly A549) that VGLL4 knockdown downregulates PD-L1 expression (seemingly moreso than in MG549, LCC is not assessed in this way except for augmentation of PD-L1 expression in the final panel), and that this seems to happen despite seemingly intact interferon signaling via STAT1 due to PD-L1 promoter-specific suppression. The authors go on to demonstrate VGLL4's interaction with IRF2BP2 via inhibiton of ubiquination and subsequent proteasome degradation (using the A549 and HEK293T lines). IRF2BP2 knockout is shown to have a modest effect on PD-L1 expression via IF2 interaction with the PD-L1 promoter in the setting of IFN stimulation in A549 cells, and YAP/miR-130a is shown

to inhibit IFN induced PD-L1 and IRF1 expression in A549 cells. Finally, the authors examine a clinical database to correlate VGLL4 with survival in lung cancer patients, and show its expression in lung cancer tissue and its association with IRF3BP2 expression. The overall thesis of the authors is certainly novel, and the level of detail in which the molecular mechanism is studied is impressive. However, there are several questions/concerns which arose during my review of the manuscript:

Major points:

- *The authors' initial animal work is thorough and compelling. However, the subsequent experiments are largely conducted on the A549 cell line (and several others). Why were these cell lines used instead of the initial LCC and MB49 lines? Was any attempt made to grow A549 in mice to recapitulate these results? Or were the various downstream experiments ever tried with the LCC/MB49 lines? While the degree of work performed is exhaustive and recapitulating many experiments with new cell lines may not be feasible, this weakness in extrapolation should be heavily addressed in the discussion section.*

(Response) We apologized for the insufficient rational. We initiated this work from investigating the molecular mechanism of how VGLL4 interacts with IRF2BP2 and regulates PD-L1 expression in HEK293T and A549 cells. VGLL4 has been showed to play as a negative regulator of lung cancer cell proliferation (Zhang et al, 2014). A549 is a commonly used human lung cancer cell line. Based on these, we decided to use A549 to perform the mechanistic study, which is more relevant to human cancer. Because A549 is a human cell line, which can only grow in immune compromised mice, such as nude mice. Murine syngeneic allograft tumor models have been well characterized and extensively used to study reciprocal interactions between tumor cells and host anti-tumor immune responses. The major findings, such as reduced PD-L1 expression and stabilization of IRF2BP2 after removal of VGLL4 are consistent in human and mouse tumor cells. We have discussed this in the discussion on page 18.

- *The downregulation of PD-L1 in response to VGLL4 knockdown was ultimately rather modest in MB49. Did the authors study its downregulation in LCC? Additionally, other T cell inhibitory factors (B7-H4) are also downregulated in response to VGLL4 knockdown. Did the authors ever study the responsiveness of these cells to inferferon-mediated cell killing? It would seem that the degree of PD-L1 downregulation might only be one contributor to the data seen in Figure 1/EV1/EV2, and it may be a more holistic reversal of T cell exclusion.*

(Response) As reviewer's suggestion, in the revised Fig. 2A, we included the gene expression analysis of immunomodulator gene in LLC cells. We found that only the downregulation of PD-L1 is consistent in LLC, MB49 and A549 cells. As the reviewer's suggestion, we also performed the experiment about the effect of IFNγ on cell survival. However, we did not observe inferferon-mediated inhibition of LLC and MB49 cell proliferation shown in revised Fig. EV2H. In addition, we performed

T-Cell mediated cell killing experiments and found that knockdown of VGLL4 significant enhanced T cell-mediated cell killing in revised Fig. 2L. We agree the reviewer's point, the degree of PD-L1 downregulation might partially contribute to the phenotype and more investigations are needed.

-Figure 1K shows only 1 field of each stain in each type of animal. Having a more quantitative measure of increased CD3/CD8/CD45 cells would be more convincing, such as cells/high-powered field (take average of multiple fields per tumor per animal). This would enable statistical comparison of the different amounts of marker-positive cells.
(Response) As reviewer's suggestion, we have quantified CD3/CD8/CD45 staining in the revised Fig. 1K .

-The methods used to generate Figure 7 are not described in the materials and methods. There is no description of what the authors used for high/low cutoffs of VGLL4 expression. Additionally, if the authors attempted a hazard ratio calculation (of risk of death) it might be more compelling than an arbitrary cutoff value and associated survival curves (although both could certainly be reported, particularly if the hazard ratio is consistent with the trend reported).
(Response) We apologized for the missing information in M&M and have included them on page 22, line 14-19. We have included the information of cutoffs of VGLL4 expression in the last lane of EV table1. The original analysis is based on hazard ratio calculation.

-The methods used to generate the IHC data in figure 7 is not described in materials and methods. Also, how did the authors quantify the degree of expression? Positive cells/high powered field would be preferable, but IHA score is another option (it is unclear how the authors scored these samples, as well as all methods for the slides' generation/staining).
(Response) We apologized for the missing information in M&M. We have included the IHC information in M&M on page 19-20. We included the score in the Fig.7A

Minor points:
-For figure EV2, the data would benefit from also showing the tumor volume curves in addition to the comparison of the tumor weight.
(Response) As reviewer's suggestion, we have included the tumor growth curve in the revised Fig. EV2F.

-For the flow cytometry data in Figure 2, representative gating would be helpful (this can be a supplemental figure).
(Response) As reviewer's suggestion, we have included the gating information of the flow cytometry data in the revised Fig. EV2A and EV2B.

-*Figure 5A - the significance between various bars in the graph should be more clearly delineated with connecting lines as in 5B and 5C.*
(Response) We apologized for the missing information of bar graph. We have revised the graph as reviewer's suggestion in the revised Fig. 5A.

-*In general, the figure legends are very brief. Unless forbidden for editorial reasons, more descriptive legends and labeling of the various cell lines used for the various experiments would greatly help the readers.*
(Response) We have revised the figure legend and the presentation of Figures as the reviewer's suggestion.

-*There are multiple minor instances where the English syntax could be improved by a copy editor.*
(Response) We have carefully revised the manuscript.

Referee #3:

The manuscript titled "Loss of VGLL4 suppresses tumor PD-L1 expression and immune evasion" by Wu et. al. show that VGLL4 deficiency in tumor cells reduces PD-L1 expression. The manuscript provides an experimental evidence for VGLL4's novel role in anti-tumor immunity. Loss of VGLL4 reduces PD-L1 expression in lung cancer cells. VGLL4 regulates PD-L1 expression by modulating IRF2/IRF2BP2 complex. VGLL4 binds IRF2BP2 and stabilizes IRF2BP2 from proteasome-mediated protein degradation. In addition, the authors show that YAP activation inhibits VGLL4 and IRF1 expression through miR-130a and supresses IFNγ-inducible PD-L1 expression. Present finding on the VGLL4 as an important regulator of PD-L1 expression is interesting and novel. There are some concerns for this manuscript.

Major
1. *How stabilization of IRFBP2 by Vgll4 sustained PD-L1 was not really addressed. Since the author speculated that IRF2 may have increased binding to PD-L1 promoter, then knockdown of IRF2 together with Vgll4 silence should restore L1 expression and tumor growth?*
(Response) We greatly appreciate the reviewer's input and believe that addressing the raised questions will improve our manuscript. As reviewer's suggestion, we silenced IRF2 together with VGLL4 in A549 cells. We found that the expression of PD-L1 was restored in double knockdown of IRF2 and VGLL4 A549 cells comparing to VGLL4 knockdown cells. This result suggests that IRF2 acts downstream of VGLL4 in regulating PD-L1 expression. Because we found that knockdown of IRF2 in LLC and MB49 cells significantly inhibited the proliferation of LLC and MB49 cells, we didn't perform the rescue experiment of knockdown of IRF2 together with depletion of Vgll4 in animal model.

2. *Over expression of Vgll4 in low expression cells should be performed and it can promote PD-L1 expression and tumor growth in immune competent but not deficient mice.*

(Response) According to the reviewer's suggestion, we overexpressed VGLL4-HF4A (do not inhibit cell growth in vitro) in B16F10 cells. We found that expression of VGLL4-HF4A promoted the B16F10 tumor growth in C57BL/6 mice, but not in nude mice (revised Fig. EV3B). Furthermore, we found that VGLL4-HF4A rescued the tumor growth of VGLl4-depleted LLC cells in C57BL/6 mice (revised Fig. 3K).

3. *Since Vgll1-3 did not interact with IRFBP2, their silence should not affect PD-L1 expression and this should be done and shown.*

(Response) According to the reviewer's suggestion, we examined the effect of VGLL1-3 on the expression of PD-L1. Because we found that there are undetectable VGLL1 and VGLL2 mRNAs in A549 cells, we only depleted VGLL3 in A549 cells. qRT-PCR analysis showed that knockdown of VGLL3 did not affect the expression of PD-L1 (revised Fig. EV2D).

4. *How YAP-suppressed IRF1 and PD-L1 expression is linked to Vgll4? Are we talking about one integrated regulation or two independent regulations? More mechanistic details are needed in addition to simply drawing a model.*

(Response) We appreciate the reviewer's input similar as reviewer 1 regarding the connection among YAP, VGLL4, IRF1 and PD-L1. We performed an additional experiment in the revised Fig. 6L. We generated a miR130a KO cell line. We found that deletion of miR-130a cannot completely release the suppression by YAP5SA, which indicate that the inhibition is in part mediated by the YAP/TEAD activity. We have toned down the role of miR130a in regulating the suppression of PD-L1 by YAP and discus this in the discussion. As we showed in the manuscript that YAP also inhibited TNFα-induced PD-L1 expression. YAP may regulate PD-L1 expression through multiple pathways. We discussed this point in the discussion on page 17. The molecular mechanism about the suppression of IFNγ-inducible PD-L1 expression by YAP needs more investigation.

5. *YAP inhibits IFNγ-inducible PD-L1 expression through miRNA-130a-mediated suppression of VGLL4 and IRF1 expression? It is important to assess the relevant contributions of Vgll4 vs IRF1?*

(Response) This question is related with the question 4. We think VGLL4 and IRF1 in parallel partially mediate the suppression of PD-L1 expression by YAP through miR-130a as discussed in question/answer 4. IRF1 is a highly potent and major inducer of PD-L1 expression. Manipulation of IRF1 expression will result in significant change of PD-L1 expression. As shown in revised Fig. 5C, expression of IRF1 still promotes PD-L1 expression in VGLL4 knockdown cells. So, VGLL4 acts upstream of IRF1 to regulate PD-L1 expression in VGLL4-IRF2BP2/IRF2-IRF1 axis.

6. *IRF1 over-expression should restore the suppression of PD-L1 imposed by Vgll4 silence or YAP overexpression as well as Tumor growth?*

(Response) According to the reviewer's suggestion, we have examined the effect of overexpression of IRF1 in VGLL4 depleted cells on PD-L1 expression. We found that overexpression of IRF1 in VGLL4-depleted A549 cells restored PD-L1 expression (revised Fig. 5C). Furthermore, overexpression of IRF1 restored the tumor growth of VGLL4-depleted LLC cells in C57BL/6 (revised Fig. EV2I). In addition, overexpression of WT-YAP decreased LLC tumor growth in murine syngeneic tumor model, not in nude mice (revised Fig. 6G). Previous study about the role of Hippo and YAP in anti-cancer immunity (Moroishi et al, 2016) indicates that the anti-cancer immunity function of Hippo-YAP may be through regulating the secretion of nucleic acid-containing extracellular vesicles and biosynthesis of microRNA (Mori et al, 2014). YAP function on immune suppression needs more studies. We did not further investigate the role of YAP in this study.

7. *A rescue with VGLL4 that is mutant to siRNA effects is required to show the restoration of PD-L1 levels in A549, and subsequent tumorigenic effects in syngeneic mice models. Also, the PDL1 levels in A549 does not seem to correlate with the level of knockdown of VGLL4 si-1 and 2 in Fig 2D. Moreover, the rationale for using A549 cells is unclear to me, when LLC cell lines have the most VGLL4 expression amongst lung cancer cells, as shown in Fig 1A.*

(Response) As reviewer's suggestion, we performed a rescue experiment. Because WT-VGLL4 greatly suppresses YAP activity and inhibits cancer cell proliferation, we used VGLL4-HF4A, a potent inducer of PD-L1 expression to rescue PD-L1 expression in VGLL4 knockdown cells. We found that VGLL4-HF4A efficiently restored the PD-L1 expression in VGLL4 depleted A549 cells (revised Fig. 3J). We have tested several siRNA and shRNA and found that all of them can deplete VGLL4 and result in the downregulation of PD-L1 in several different cell lines.

We apologized for the insufficient rational. Reviewer 3 raised the similar question as reviewer 2. We initiated this work from investigating the molecular mechanism of how VGLL4 interacts with IRF2BP2 and regulates PD-L1 expression in HEK293T and A549 cells. VGLL4 has been showed to play as a negative regulator of lung cancer cell proliferation (Zhang et al, 2014). A549 is a commonly used human lung cancer cell line. Based on these, we decided to use A549 to perform the mechanistic study, which is more relevant to human cancer. Murine syngeneic allograft tumor models have been well characterized and extensively used to study reciprocal interactions between tumor cells and host anti-tumor immune responses. The major findings, such as reduced PD-L1 expression and stabilization of IRF2BP2 after removal of VGLL4 are consistent in human and mouse tumor cells. We have discussed this in the discussion on page 18.

8. *The authors interpret that TDU domains are not required for IRF2BP2-VGLL4 interactions; however, at later stages (Fig 6), their data imply that binding of YAP-TEAD controls PDL1 expression under IFN response. In that case, what is the*

status of VGLL4-TEAD versus TEAD-YAP complexes in these cell types with or without IFN induction? In other words, which partnering complex is more prevalent upon IFN induction?

(Response) According to reviewer's suggestion, we performed co-IP experiment to examine the interaction of TEAD with YAP or VGLL4 under IFNγ treatment. We found that IFNγ treatment did not affect the interaction of TEAD with YAP or VGLL4 (revised Fig. EV5A).

9. *A knockdown of TEAD in the VGLL4 kd A549 cells is recommended to denote its effects on IRF2BP2 and PDL1, respectively.*

(Response) According to reviewer's suggestion, we performed a new experiment to deplete TEADs in A549 cells to examine the status of IRF2BP2 and PD-L1 in the revised Fig. EV4A and EV4B. We found that there were increased IRF2BP2 proteins in the TEADs-depleted A549 cells. We reasoned that due to the depletion of TEADs, more VGLL4 proteins were released from TEADs/VGLL4 complex and bound to IRF2BP2 proteins to stabilize IRF2BP2 proteins. However, we did not observe the increased PD-L1 expression, which may be due to the decreased TEADs/YAP transcriptional activity in TEADs knockdown cells.

10. *What is the status of YAP expression and their targets, and miR130a, in A549 cells that were depleted of VGLL4? The expression of YAP and its targets also needs to be studied in VGLL4 deficient tumors that were rescued by mouse-PDL1.*

(Response) We have examined the miR-130a level in VGLL4 knockdown A549 cells and found a slightly increased miR-130a expression in VGLL4 depleted A549 cells (revised Fig. EV6A). We also examined YAP target gene expression in PD-L1 overexpressing VGLL4 depleted LLC tumors and found that the expression of PD-L1 did not affect YAP target gene expression (revised Fig. EV2G).

11. *Despite robust effects on VGLL4, I do not see a corresponding significant effect of miR130-a mimic or sponge in down- and up-regulating PDL1 expression (Fig 6a-b). For instance, in panel b, the levels of VGLL4 are strongly up by 12h post IFN induction, whereas PDL1 levels are not. This gives the reader an impression that miR130a route may not be the major regulator of PDL1 even though it may regulate VGLL4.*

(Response) We performed a new experiment further investigate the role of miR-130a in the revised Fig. 6L. We generated a miR-130a KO cell line, in which miR-130a is completely deleted rather than downregulated. We found that deletion of miR-130a cannot completely release the suppression by YAP5SA, which indicate that the inhibition is in part mediated by the YAP transcriptional activity. Interestingly, one study reported that IFNγ-inducible expression of PD-L1 was dependent on NF-κB (Gowrishankar et al, 2015). In our study, we showed that YAP also suppressed TNFα-inducible PD-L1 expression through inhibiting NF-κB signaling, which did not require the transcriptional activity of YAP. Alternatively, YAP may regulate IFNγ-inducible expression of PD-L1 through NF-κB signaling. The detailed

mechanism of the crosstalk between Hippo-YAP and TNFα-NF-κB will be published elsewhere. We have toned down the role of miR-130a in regulating the suppression of PD-L1 by YAP and discuss this in the discussion on page 17.

12. *Why was the expression pattern of PDL1 not included in the lung cancer specimens in Fig 7A? Also, what is the YAP expression profile in these lung cancer specimens that have high VGLL4-IRF2BP2 expression?*
(Response) We have performed the analysis as reviewer's suggestion. However, we didn't observe a significant correlation of VGLLL4 and YAP expression (revised Fig. EV7A), which indicates that a more complex regulation in the clinical samples. We showed that there is a weak correlation between VGLL4 and PD-L1 expression (revised Fig. 7C). Because PD-L1 expression is often clustered rather than uniformly diffuse in tumor tissues and is likely localized to the area where IFNγ^+ T cells infiltrate (Ribas & Hu-Lieskovan, 2016; Zou et al, 2016). Thus, human tumor tissue array may miss the PD-L1–positive area and give false-negative results, or contain a high T cell infiltrate area and give false-positive results. We included this discussion in the revised manuscript on page 15, line 18-23.

2nd Editorial Decision 25th Sep 2018

Thank you for sending us the revised version. Your study has now been re-reviewed by the three referees and the comments are provided below. As you can see from the comments, the referees appreciates the introduced changes and support publication here.

There are just some remaining editorial issues to be sorted before we can send you the formal acceptance letter.

Could you please comment on the concern raised by referee #2 regarding the IHC scoring system used and make sure this is well described how this was done?

REFEREE REPORTS:

Referee #1:

I have carefully examined the impressive revision efforts of the authors and came to the conclusion that they have adequately addressed all my major and minor concerns.
Thus, I recommend to accept this manuscript for publication in the EMBO Journal.

Referee #2:

The manuscript authors have revised their work and have addressed most of my critiques of the original draft. I am somewhat hesitant about the use of an entirely subjective IHC scoring system which has not been validated in Figure 7. However, I recognize thatfully quantitative or semi-quantitative measures may be beyond the scope of this work given how it fits in with the general narrative and other experiments. The English syntax still has some areas where it could be greatly improved. However, I will defer to the editor on both points, and the remainder of my original critiques have been adequately addressed.

Referee #3:

am happy with the revisions

2nd Revision - authors' response 27th Sep 18

We would like to thank the reviewers for their positive comments.

Referee #1:
I have carefully examined the impressive revision efforts of the authors and came to the conclusion that they have adequately addressed all my major and minor concerns. Thus, I recommend to accept this manuscript for publication in the EMBO Journal.

Referee #2:
The manuscript authors have revised their work and have addressed most of my critiques of the original draft. I am somewhat hesitant about the use of an entirely subjective IHC scoring system which has not been validated in Figure 7. However, I recognize that fully quantitative or semi-quantitative measures may be beyond the scope of this work given how it fits in with the general narrative and other experiments. The English syntax still has some areas where it could be greatly improved. However, I will defer to the editor on both points, and the remainder of my original critiques have been adequately addressed.

Response: We agree the reviewer's point that quantitative or semi-quantitative measures, such as western blot of fresh clinical tumor samples, are better than the subjective IHC scoring system of paraffin tissue arrays. Reviewer also pointed out that the quantitative or semi-quantitative measures are beyond the scope of this work. We added a sentence "*Alternatively，quantitative or semi-quantitative measures, such as western blot of fresh clinical tumor samples will give a more precise result.*" in the discussion to reflect the reviewer's point on page 15, line 25-26.
For the English syntax, we have carefully revised the manuscript.

Referee #3:
I am happy with the revisions.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Hai Song
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99506R

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 $\chi 2$ 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?	All experiments were performed with at least three independent samples and data reported as means with representitative images. the number of mice used in the statistical analysis was more than the minimum number of mice according the guidelines for the use of experimental animals.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have stated that "No statistical method was used to predetermine sample size in the animal studies." in the Materials and Methods.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
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.	Animals were randomly assigned to the cancer cell transplantation groups.All mice, samples and data were processed in the same manner indepemdent of the experimental condition.
For animal studies, include a statement about randomization even if no randomization was used.	We have stated that "No statistical method was used to predetermine sample size in the animal studies." in the Materials and 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.	Not applicable
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No such methods were used.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were in good conditions but were not tested for mycoplasma contamination.

* 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.	C57BL/6 and nude mice were purchased from Shanghai SLAC Laboratory Animal Company. 5- to 10-week-old mice were used all animal experiments.Mice were housed under SPF conditions in the enviroment of 20-22 ℃, with a 12/12 hours light and dark cycle, 50-70% humidity.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal studies were approved by the Zhejiang University Animal Care and Use Committee.
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.	We confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

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	Not applicable
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).	Not applicable
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).	Not applicable
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.	Not applicable

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.	Not applicable