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TGIF1 functions as a tumor suppressor in pancreatic ductal adenocarcinoma

Parash Parajuli, Purba Singh, Zhe Wang, Lianna Li, Sailaja Eragamreddi, Seval Ozkan, Olivier Ferrigno, Celine Prunier, Mohammed S. Razzaque, Keli Xu and Azeddine Atfi

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(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 Dec 2018

Thank you for the submission of your manuscript (EMBOJ-2018-101067) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your work, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. Referee #2 states that the downstream targets of TGIF-Twist1 remain unclear and the link to TGFbeta is not sufficiently supported by the data in his-her view. Further, this referee states that the pathophysiological relevance of the proposed signaling axis would need to be more rigorously addressed. Referee #3 agrees that global analyses should be performed to clarify downstream pathways (ref#3 pt. 4). In addition, the referees point to issues related to additional controls needed and data illustration would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS:

Referee #1:

This manuscript by Parajuli et al. examines the role of TGIF in the development of pancreatic ductal cancer (PDAC). The authors report that TGIF acts as a tumor suppressor in the context of oncogenic Kras (KrasG12D) driven PDACs. TGIF interacts with Twist1 protein and represses Twist transcriptional activation by binding its promoter. Inactivation of TGIF via phosphorylation leads to

an increase in Twist expression, an important effector of oncogenic Kras initiated PDACs. The authors conclude from their studies that TGIF acts to oppose Twist-1 mediated cancer progression and may lead to novel therapeutic strategies for PDAC.
The quality of the study is excellent and conclusions are supported by the data presented. As currently presented, additional experiments are warranted to strengthen the conclusions of the study.

Figure 1: Arrows pointing to PanINs in KC sections of panel 1E would be helpful here.
Figure 2: Panel C demonstrates metastatic burden in KTCLuc mice, the figure legends state that n=6 mice were used in this study, what is the % of these mice that developed metastasis of brain, liver and lung.
Figure 3: Yeast 2 hybrid data indicates that the interaction between TGIF and Twist proteins is direct as opposed to a result of their association at the Twist promoter. DNase treated Co-IP samples should be included in this panel to strengthen these findings.
In panel 3A, MIAPaCa-2 cells are utilized which harbor a constitutively active kRas, a comparison with a WT kRas line would bolster these findings. This would also be useful for luciferase reporter experiments and for pTGIF blots.
Figure 4: Clarification of the TwistER vector used in panel A is needed, should tamoxifen treatment lead to an induction of TwistER expression here? In addition, the primers used to amplify Twist1 are in the ORF of the gene, how are endogenous Twist and TwistER transcript distinguished in this panel?
Figure 6: Do the mutant kRas cell lines used here in panel 6C and throughout the paper exhibit a more epithelial or mesenchymal phenotype? What are the endogenous levels of TGIF and Twist1 like in this panel?
In panel 6A and 6C: a double band is observed for WT Flag TGIF samples, this doublet is also observed in TGIF blots in Figure 3, could the authors comment on the presence of this doublet in the results section.
Panel 6B: pTGIF1 levels were assessed in TMAs including 183 human PDAC samples. Only representative images are shown here. Scoring and statistical analyses of the complete dataset should be included. A correlation between Twist1 and pTGIF1 expression in these samples would significantly strengthen the findings of this study.
EV3: The authors conclude that activation of TGFβ signaling had little or no effect on the physical interaction between TGIF and Twist1. A treatment time course is needed to substantiate this conclusion.
Minor issues:
The line representing TgifKO is not visible in the KM plot of Fig 1C
Typographical error:
page 4 of discussion "activation of twist1 independently"
Real-time PCR section of methods: human Twist11-Rev primer

Referee #2:

Parajuli and co-workers report the intriguing finding that the pancreas-specific genetic depletion of Tgif1 results into an acceleration of malignant tumor progression and metastasis in the KC transgenic mouse model of PDAC. They go on to unravel the molecular mechanisms underpinning Tgif1's tumor suppressive function. Using a combination of histopathological, cell biological and biochemical experimentation they demonstrate that Tgif1 interacts with Twist1 and thereby prevents Twist1's transcriptional repression of E-cadherin and p16 and of its own expression. The experiments are presented in a concise and clear manner, they are well controlled, and the results widely support the conclusions drawn by the authors. In fact, some of the experiments could serve as role models for the elegant combination of gain and loss function experiments and functional experimentation.

When it comes to detailing the functional contribution of Tgif1 to PDAC progression, the data is highly conclusive, although still limited when it comes to PDAC progression in patients. The molecular mechanisms unraveled by experimental work and presented in the manuscript are novel and convincingly support the conclusions of the tumor suppressive function of Tgif1 in PDAC and

they provide exciting new insights into the functional contribution of Tgif1 and Twist1 in PDAC progression.

However, the authors also claim that Tgif1's function may explain the dual role of TGFbeta signaling in cancer in general. Here, the authors stop short in making the point. Such mechanistic explanation has not been directly addressed in the manuscript, and the role of Tgif1 - as it presented - only partially (and only selectively) contributes new insights into this enigma of cancer research. Notably, the authors have handpicked two downstream effector genes and have missed the opportunity to perform a transcriptome-wide comprehensive analysis of the functional contribution of the interaction between Tgif1 and Twist1 to malignant tumor progression, for example by RNA sequencing and by ChIP-sequencing experiments. Identification of the effector genes and their combined gene expression signature may also provide tools to characterize or predict PDAC progression in patients. Finally, while the authors demonstrate that Tgif1 affects Smad phosphorylation, they do not experimentally connect Tgif1/Twist1 with TGFbeta signaling. Along these lines, it is noted that the authors do not mention the distinction between canonical vs. non-canonical TGFbeta signaling pathways which have been shown to contribute to the TGFbeta's dual role in cancerogenesis. Shouldn't the KC-Tgif1 KO mice be compared to KC-Smad4 KO mice?

Another point which needs clarification is the fact that the authors conclude that MAPK induced by constitutive-active Ras phosphorylates Tgif1 and thus represses its tumor suppressive function (see model of Figure 7). Yet, Ras is also active in the KC mouse model which fails to rapidly progress, but is dramatically accelerated by the depletion of Tgif1. Along these lines, non-canonical TGFbeta signaling also involves MAPK signaling and thus could replace Ras-induced MAPK signaling? Moreover, could a MEK inhibitor mimic Tgif1 function and repress PDAC progression in the mouse models used?

Minor comments:

Figure 1B: the magnification of the panels in Control and Tgif1 KO seems to be different.

Size bars are missing throughout.

Figure 1D: the spleens are pale in Tgif1 KO mice. Any explanation?

Figure EV2C: the immunohistochemical staining for Junb is not very convincing (in quality).

The chapter heading: Tgif1 "promotes" PDAC formation.... should say ...."represses"....

The tissue microarray for p-Tgif1 histochemistry of patient samples should be statistically evaluated and correlated with PDAC staging/grading.

Referee #3:

General Summary:

The manuscript by Parajuli et al describes the role of TGIF1 as a tumor suppressor in PDAC having KRAS mutation. Using CoIP and ChIP assay authors confirmed the ability of TGIF to suppress PDAC through inhibition of TWIST1 expression and its interaction with TGIF1. Further, this manuscript provides insight into mechanistic details on how TGIF1 inhibits progression of metastasis in PDACs. The authors described the importance of TGIF1 phosphorylation as an inhibitor of its anti-tumor activity in human PDAC harboring constitutive KRAS signaling.

Overall significance of the study:
This manuscript adds new and interesting findings that will strengthen the understanding of novel role of TGIF1 in molecular events leading to progression of PDAC with KRAS mutation. Overall

the manuscript is very well written and most of the results are substantiated with proper controls, description and references. The manuscript in the current status can be considered for publication provided the authors will address the following corrections and concerns:

Minor concerns:

1) In general, for all IHC images magnification and scale bar should be provided.
2) In case of figure 2A the pancreas size highlighted in KC mice is different from what is shown in panel 1D of figure1. Is there any reason for this or it is reflection of the way the picture is taken thus hiding the rest of the pancreas? Secondly, since the tumor in the KTC mice is bigger than the KC mice this may predict more metastasis observed in KTC mice.
3) In figure 2D loading control has to be incorporated to validate the findings.
4) In figure 3 panel (D and E), right panel looks like representation of densitometric analysis of the left panel or if it represents independent experiment has to be made clear in the figure legend. Secondly, it would be good to show only one panel here and move the other panel in the supplementary figures.
5) For figure 4G it would be important to show the IHC panel for p16 protein also along with E-cadherin and vimentin.
6) In case of Figure 5 expression of cdh1, vimentin and p16 has been shown at transcript level however, it would be a good to include IHC/western blot assay for the same to show expression at protein levels to keep it consistent throughout the manuscript.
7) In case of figure 6 stable cell lines were generated expressing TGIF1 and mutant of TGIF1 and expression of same are shown in panel C however, the respective empty vehicle panel and loading control is not shown for this panel.
8) The results state that Twist1 expression depends only on the level of TGIF1 phosphorylation and not on quantity of TGIF1 protein. Thus, the model on Fig. 7 would better represent the results if the amount of TGIF1 "rectangles" on the left part of model will be the same as on the right part but only (e.g. 1 of 3) will be marked as phosphorylated.

Major concerns:

1. For figure 2B lymph node panel describing metastasis in KTC mice should be validated by staining with some markers of invasion.
2. In figure 3A Input for the CoIP experiment is not presented and for panel C densitometric analysis should be provided. Reciprocal CoIP for the same is missing to further validate the data. For figure 2B in case of TGIF KO panel because of the absence of TGIF in input as it is knock out mice therefore, it is obvious to not find interaction between Twist1 and TGIF1. The best way to prove this point will be to use an inducible system and show that interaction persist in the absence of inducer and diminish in presence of inducer.
3. CoIP or protein interaction assays should be performed to show the effect on the association of twist1 with TGIF1 when its phosphorylated. Further, effect of TGIF1 phosphorylation on the expression of TWIST1 target genes should be shown to underscore the importance of TGIF1 phosphorylation function in disrupting its anti-tumor activity. Along the same line, it should be checked that whether specific inhibitor for phosphorylation can revert the observations.
4. It would be of due importance to go for RNA-seq/micro-array in TFIG KO/KC/KTC conditions to identify the genes and pathways that gets modulated. This experiment can provide insight into some new targets and pathways related to EMT or TGF-beta/smad signaling or may be wnt signaling considering TGIF1 has a role to play in wnt-signaling and will strengthen the impact of this study.

1st Revision - authors' response 3rd Mar 2019

Reviewer 1

This manuscript by Parajuli et al. examines the role of TGIF in the development of pancreatic ductal cancer (PDAC). The authors report that TGIF acts as a tumor suppressor in the context of oncogenic Kras (KrasG12D) driven PDACs. TGIF interacts with Twist1 protein and represses Twist transcriptional activation by binding its promoter. Inactivation of TGIF via phosphorylation leads to an increase in Twist expression, an important effector of oncogenic Kras initiated PDACs. The

authors conclude from their studies that TGIF acts to oppose Twist-1 mediated cancer progression and may lead to novel therapeutic strategies for PDAC.

The quality of the study is excellent and conclusions are supported by the data presented. As currently presented, additional experiments are warranted to strengthen the conclusions of the study.

Reviewer 1
Figure 1: Arrows pointing to PanINs in KC sections of panel 1E would be helpful here.

Authors
We added these arrows in Fig.1E (also described in Legends to Figures/page45).

Reviewer 1
Figure 2: Panel C demonstrates metastatic burden in KTC-Luc mice, the figure legends state that n=6 mice were used in this study, what is the % of these mice that developed metastasis of brain, liver and lung.

Authors
Five out of six in KTC^{Luc} mice developed metastasis to the liver and lung, whereas none of the six mice developed metastasis to the brain. This observation is discussed in the manuscript (Results/page11/paragraph1 and Legend to Figures/page46).

Reviewer 1
Figure 3: Yeast 2 hybrid data indicates that the interaction between TGIF and Twist proteins is direct as opposed to a result of their association at the Twist promoter. DNase treated Co-IP samples should be included in this panel to strengthen these findings.

Authors
We did Co-IP with samples treated with DNase I, and found that TGIF1 associated with Twist1 in both the absence and presence of chromatin (Fig 3C). This result is discussed in the revised manuscript (Results/page12/paragraph1).

Reviewer 1
In panel 3A, MIAPaCa-2 cells are utilized which harbor a constitutively active Kras; a comparison with a WT kRas line would bolster these findings. This would also be useful for luciferase reporter experiments and for pTGIF blots.

Authors
As suggested by the Reviewer, we used the BxPC3 cell line, the only human PDAC cell line reported to harbor wild-type Kras, to the best of our knowledge. We found that TGIF1 is more phosphorylated in MIAPaCa-2 cells than in BxPC3 cells (Fig EV6A), consistent with our model. This result is discussed in the revised manuscript (Results/page16/paragraph2).

Unluckily, we found that the expression of Twist1 in BxPC3 cells is low, hampering any attempt to use this human PDAC cell line in coimmunoprecipitation assays to detect the association of endogenous TGIF1 and Twist1. Further, the moderate transfection efficiency did not allow us to conduct coimmunoprecipitation with overexpressed proteins. As an alternative approach to overcome this limitation, we sought to use the mammalian two-hybrid system (described in our previous publications, *Cell Reports* 4:530-541, 2013 and *Cancer Cell* 27:547-560, 2015), which proved to be a more sensitive method to analyze the interaction of TGIF1 with Twist1 in BxPC3 cells co-transfected with $Kras^{G12D}$ to trigger TGIF1 phosphorylation. As anticipated from our coimmunoprecipitation experiments, we detected a specific and robust interaction of TGIF1 with Twist1 in cells cotransfected with empty vector (Fig EV6C). More importantly, that interaction was decreased when $Kras^{G12D}$ was cotransfected (Fig EV6C). Under these experimental conditions, TGIF1.2TA (phosphorylation-defective mutant) interacted more strongly with Twist1, and this interaction was not affected by $Kras^{G12D}$ (Fig EV6C). This result is discussed in the revised manuscript (Results/page17/paragraph2).

In attempts to corroborate these findings, we found that $Kras^{G12D}$ expression blunted the ability of wild-type TGIF1 to repress Twist1 expression (Fig 6F), as assessed by our gene porter assay using TGT^{Luc} (luciferase driven by the Twist1 promoter). In marked contrast, $Kras^{G12D}$ did not

interfere with the ability of TGIF1.2TA to repress Twist1 expression (Fig 6F). This result is discussed in the revised manuscript (Results/page17/paragraph3).

Together, these findings confirm our original observation that oncogenic Kras inhibits TGIF1 activity towards Twist1, likely owing to its phosphorylation by ERK/MAPK.

Reviewer 1
Figure 4: Clarification of the $Twist^{ER}$ vector used in panel A is needed, should tamoxifen treatment lead to an induction of $Twist^{ER}$ expression here? In addition, the primers used to amplify Twist1 are in the ORF of the gene, how are endogenous Twist and TwistER transcript distinguished in this panel?

Authors
We consistently observed a slight increase of Twist1 expression upon Tamoxifen stimulation. The mechanism behind this phenomenon is unclear. Because Tamoxifen binding allows translocation of $Twist1^{ER}$ to the nucleus, one would speculate that the nuclear Twist1 might be less accessible for degradation than the cytoplasmic Twist1. This possibility is discussed in the revised manuscript (Results/page13/paragraph2).

The $Twist1^{ER}$ construct encodes mouse Twist1 fused to the estrogen receptor. The primers used to amplify human Twist1 in the human PDAC cell line PL45 cells do not recognize mouse Twist1, thus enabling us to discriminate between endogenous human Twist1 and $Twist1^{ER}$. This information is included in the revised manuscript (Materials and Methods/page34/paragraph3).

Reviewer 1
Figure 6: Do the mutant kRas cell lines used here in panel 6C and throughout the paper exhibit a more epithelial or mesenchymal phenotype? What are the endogenous levels of TGIF and Twist1 like in this panel?

Authors
Suit-2 and MIAPaCa-2 cells display a more mesenchymal phenotype, whereas Capan-2 and Panc-1 cells display a more epithelial phenotype (Fig 7A). We found that Twist1 is more expressed in Suit-2 and MIAPaCa-2 cells than in Capan-2 and Panc-1 cells, and this was inversely correlating with TGIF1 expression (Fig 7B). This result is discussed in the revised manuscript (Results/page18/paragraph1).

Reviewer 1
In panel 6A and 6C: a double band is observed for WT Flag TGIF samples, this doublet is also observed in TGIF blots in Figure 3, could the authors comment on the presence of this doublet in the results section.

Authors
TGIF1 migrates as a double band in the SDS-PAGE. The slower-migrating band corresponds to the phosphorylated form, whereas the faster-migrating band corresponds to the non-phosphorylated form. This likely explains why in Fig 6A and Fig 6C (now Fig 7C), the slower-migrating band was converted to the faster-migrating band when the TGIF1 phosphorylation sites were mutated. This information is indicated in the revised manuscript (Results/page16/paragraph2).

Reviewer 1
Panel 6B: pTGIF1 levels were assessed in TMAs including 183 human PDAC samples. Only representative images are shown here. Scoring and statistical analyses of the complete dataset should be included. A correlation between Twist1 and pTGIF1 expression in these samples would significantly strengthen the findings of this study.

Authors
As suggested by the Reviewer, we included scoring data (Fig EV6B). In addition, we analyzed Twist1 expression in the human TMA, and found that high TGIF1 phosphorylation was associated with high Twist1 expression and vice-versa (Figs 6B and 6C). These data are discussed in the revised manuscript (Results/page17/paragraph1).

Reviewer 1

EV3: The authors conclude that activation of TGFβ signaling had little or no effect on the physical interaction between TGIF and Twist1. A treatment time course is needed to substantiate this conclusion.

Authors
We performed this time course experiment, and found that activation of TGF-b activation for increasing time up to 8 hours had little or no effect on the association of TGIF1 with Twist1 (Fig EV3B). This result is discussed in the revised manuscript (Results/page12/paragraph1).

Reviewer 1: Minor issues:

Reviewer 1: The line representing TgifKO is not visible in the KM plot of Fig 1C

Author
The line of *Tgif1*^{KO} was present but masked by the black line corresponding to control mice. To address this issue, we used a regular mosaic two-colors line to show the survival of control and *Tgif1*^{KO} mice simultaneously (Fig 1C). In addition, we included a graph (Fig EV1J) to show that 100% of both control and *Tgif1*^{KO} mice survived during the observation period. This result is discussed in the revised manuscript (Results/page9/paragraph1).

Reviewer 1
Typographical error: page 4 of discussion "activation of twist1 independently"

Author
This typo was corrected.

Reviewer 1
Real-time PCR section of methods: human Twist11-Rev primer

Author
This typo was corrected. We are deeply grateful to the Reviewer for pointing out these typos as well as all other constructive critiques that greatly improved the message of our manuscript. Thank you very much.

Reviewer 2

Reviewer 2
Parajuli and co-workers report the intriguing finding that the pancreas-specific genetic depletion of Tgif1 results into an acceleration of malignant tumor progression and metastasis in the KC transgenic mouse model of PDAC. They go on to unravel the molecular mechanisms underpinning Tgif1's tumor suppressive function. Using a combination of histopathological, cell biological and biochemical experimentation they demonstrate that Tgif1 interacts with Twist1 and thereby prevents Twist1's transcriptional repression of E-cadherin and p16 and of its own expression. The experiments are presented in a concise and clear manner, they are well controlled, and the results widely support the conclusions drawn by the authors. In fact, some of the experiments could serve as role models for the elegant combination of gain and loss function experiments and functional experimentation.

When it comes to detailing the functional contribution of Tgif1 to PDAC progression, the data is highly conclusive, although still limited when it comes to PDAC progression in patients. The molecular mechanisms unraveled by experimental work and presented in the manuscript are novel and convincingly support the conclusions of the tumor suppressive function of Tgif1 in PDAC and they provide exciting new insights into the functional contribution of Tgif1 and Twist1 in PDAC progression.

However, the authors also claim that Tgif1's function may explain the dual role of TGFbeta

signaling in cancer in general. Here, the authors stop short in making the point. Such mechanistic explanation has not been directly addressed in the manuscript, and the role of Tgif1 - as it presented - only partially (and only selectively) contributes new insights into this enigma of cancer research. Notably, the authors have handpicked two downstream effector genes and have missed the opportunity to perform a transcriptome-wide comprehensive analysis of the functional contribution of the interaction between Tgif1 and Twist1 to malignant tumor progression, for example by RNA sequencing and by ChIP-sequencing experiments. Identification of the effector genes and their combined gene expression signature may also provide tools to characterize or predict PDAC progression in patients. Finally, while the authors demonstrate that Tgif1 affects Smad phosphorylation, they do not experimentally connect Tgif1/Twist1 with TGFbeta signaling. Along these lines, it is noted that the authors do not mention the distinction between canonical vs. non-canonical TGFbeta signaling pathways which have been shown to contribute to the TGFbeta's dual role in cancerogenesis. Shouldn't the KC-Tgif1 KO mice be compared to KC-Smad4 KO mice?

Authors
We deeply appreciate all the agreeable and valuable comments that greatly improved the message of our manuscript. Thank you very much.

In response to these constructive critiques:

-Previous studies from our lab have shown that TGIF1 behaves as an oncoprotein in breast cancer and promyelocitic leukemia. Our present study suggests that TGIF1 might function as a tumor suppressor gene. We agree with the Reviewer that we have not presented any mechanistic data to claim that this new TGIF1's function explains the dual role of TGF-b signaling in cancer. Stating in the discussion of our manuscript that TGIF1 promotes breast cancer and promyelocitic leukemia progression was aimed to provide the readers with a general overview of how TGIF1 functions in different types of cancer. We have modified the text in our revised discussion, now stating that it would be appealing in future studies to examine whether TGIF1 phosphorylation could contribute to its role as an oncoprotein in breast cancer and acute promoyelocytic leukemia (Discussion/page24/paragraph2). We also slightly modified the text throughout the manuscript to remove any confusion regarding the hypothesis that TGIF1 might mediate the TGF-b bimodal function during cancer progression.

-For RNA sequencing and ChIP-sequencing, we do not have enough materials to conduct those experiments. Generating new *Tgif1*^{KO}, *KC*, *KTC*, and *KTWC* mice that develop PDAC (or no) will require a minimum of 15 months since we keep all our colonies with individual transgenes separated in order to facilitate other undergoing studies and also avoid unnecessary pain to mice due to possible development of PDAC. Although we were not able to present these RNA and ChIP sequencing data, the genetic and epigenetic alterations in human PDAC have been extensively characterized in many integrative genome and transcriptome studies performed by The Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC), and other consortiums or independent laboratories. In contrast, the roles of posttranslational modifications in PDAC remain poorly investigated. The fact that TGIF1's function is mainly regulated by phosphorylation provided us with a unique opportunity to investigate whether this previously uncharacterized posttranslational event could impact TGIF1's functional interactions with other proteins that might play a determinant role in PDAC. To probe this possibility, we choose to perform an integrative protein-protein interaction study by means of the yeast two-hybrid system using a general cDNA library that encompasses the vast majority of genes expressed in mammalian cells. To be focused, we decided to characterize the interaction of TGIF1 with one of the top candidates, the pro-malignant transcription factor Twist1, keeping in mind the stringent criteria that such choice must endowed us with the capability to use Twist1 conditional mice in genetic approaches to provide compelling evidence as to whether TGIF1 exerts its tumor suppressor function in PDAC by a molecular mechanism involving Twist1, despite being expected to be excessively time-consuming. With that being said, we totally agree with the Reviewer that it would be more informative to use other integrative approaches, such as RNA sequencing and ChIP-sequencing, to identify other genes and pathways that play causative roles in PDAC. Taking into consideration this highly Reviewer's constructive comment, we are definitely planning to generate again *Tgif1*^{KO}, *KC*, *KTC*, and *KTWC* mice deleted of *p16Ink4a* or *Cdh1* to conduct RNA sequencing, ChIP-sequencing, proteomic, and lipodomic experiments. It is our sincere hope to be able to provide the PDAC field in the future with additional

observations that could be exploited both in terms of understanding the mechanistic underpinnings of PDAC, and in terms of translational opportunities. We discussed these possibilities in the revised manuscript (Discussion/page22/paragraph1).

-We presented data in the revised manuscript showing that the TGIF1/Twist1 interaction occurs in a manner independent of TGF-b signaling (Figs 3D and EV3B). Based on this result, we suggested in the "Discussion" section that *Tgif1* inactivation might impact at least two distinct networks (i.e., TGF-b, Twist1) that perhaps converge together to deepen PDAC proliferative and metastatic behaviors (Results/page12/paragraph1; Discussion/page22/paragraph1).

-As suggested by the Reviewer, we discussed the non-canonical TGF-b signaling in the manuscript, especially how its activation might affect TGIF1 phosphorylation and thereby its tumor suppressor function in PDAC (Discussion/page24/paragraph2).

-Deletion of *Tgif1* led to the acceleration of $Kras^{G12D}$-driven PDAC, as does deletion of *Smad4* (for example please see the paper published in *Genes and Developmen*t, 20:3130-3146). However, *Smad4* deletion was not associated with PDAC metastasis, as opposed to *Tgif1* deletion, which fits well with the general notion that increased TGF-b signaling (as occurred in *KTC* mice) might promote cell invasion and metastasis. We discussed these possibilities in the revised manuscript (Introduction/page4/paragraph1 and Result/page8/paragraph2).

Reviewer 2
Another point which needs clarification is the fact that the authors conclude that MAPK induced by constitutive-active Ras phosphorylates Tgif1 and thus represses its tumor suppressive function (see model of Figure 7). Yet, Ras is also active in the KC mouse model which fails to rapidly progress, but is dramatically accelerated by the depletion of Tgif1. Along these lines, non-canonical TGFbeta signaling also involves MAPK signaling and thus could replace Ras-induced MAPK signaling? Moreover, could a MEK inhibitor mimic Tgif1 function and repress PDAC progression in the mouse models used?

Authors
-We found that TGIF1 phosphorylation increased at late stages of human PDAC (Figs 6B and EV6B). Based on this observation, it is possible that other genetic or epigenetic alterations might have accumulated overtime that cooperate with $Kras^{G12D}$ to activate MAPK/ERK, thereby increasing TGIF1 phosphorylation. One of these alterations could be activation of the non-canonical TGF-b signaling involving MAPK/ERK, since our present study as well as other published studies (cited in the manuscript) have shown that TGF-b is highly expressed at late stages of PDAC progression (Figs EV1H and EV1I). This possibility is discussed in the revised manuscript (Discussion/page24/paragraph2).

-Previous studies using a mouse model of $Kras^{G12}$D-driven PDAC have shown that a MEK inhibitor can suppress PDAC progression in vivo (Collins et al., Gastroenterology 146: 822-837, 2014), which is agreement with our model in which non-phosphorylated TGIF1 restricts $Kras^{G12D}$-driven PDAC. We discussed this study in the revised manuscript (Discussion/page24paragraph2).

Reviewer 2
Minor comments:

Reviewer 2
Figure 1B: the magnification of the panels in Control and Tgif1 KO seems to be different.

Authors
This mistake was corrected. The conclusion of the experiment remains unchanged.

Reviewer 2
Size bars are missing throughout.

Authors

We included size bars in all figures.

Reviewer 2
Figure 1D: the spleens are pale in Tgif1.KO mice. Any explanation?

Authors
The difference in the color of spleen might be due to the intensity of the surrounding light during the photo acquisition. We never saw any difference in color of the spleen of $Tgif1^{KO}$ mice when compared to the other mice under study (n>100 mice). We used another picture in order to keep our presentation homogeneous.

Reviewer 2
Figure EV2C: the immunohistochemical staining for Junb is not very convincing (in quality).

Authors
We conducted additional immunohistochemistry experiments to show that JunB expression was increased in *KTC* mice relative to *KC* or control mice (Fig EV2C). We confirmed these data using both immunoblotting and qRT-PCR approaches (Figs 2D and 2E). These results are discussed in the revised manuscript (Results/page11/paragraph2).

Reviewer 2
The chapter heading: Tgif1 "promotes" PDAC formation.... should say ...."represses"....

Authors
We corrected this error.

Reviewer 2
The tissue microarray for p-Tgif1 histochemistry of patient samples should be statistically evaluated and correlated with PDAC staging/grading.

Authors
We evaluated the tissue microarray for pTGIF1, and the results showed that high levels of pTGIF1 was increased during PDAC progression (Fig EV6B). These results are discussed in the revised manuscript (Results/page17/paragraph1).

Reviewer 3

General Summary

The manuscript by Parajuli et al describes the role of TGIF1 as a tumor suppressor in PDAC having KRAS mutation. Using CoIP and ChIP assay authors confirmed the ability of TGIF to suppress PDAC through inhibition of TWIST1 expression and its interaction with TGIF1. Further, this manuscript provides insight into mechanistic details on how TGIF1 inhibits progression of metastasis in PDACs. The authors described the importance of TGIF1 phosphorylation as an inhibitor of its anti-tumor activity in human PDAC harboring constitutive KRAS signaling.

Overall significance of the study: This manuscript adds new and interesting findings that will strengthen the understanding of novel role of TGIF1 in molecular events leading to progression of PDAC with KRAS mutation. Overall the manuscript is very well written and most of the results are substantiated with proper controls, description and references. The manuscript in the current status can be considered for publication provided the authors will address the following corrections and concerns:

Minor concerns:

Reviewer 3
In general, for all IHC images magnification and scale bar should be provided.

Authors

We added magnifications and size bars in all figures.

Reviewer 3
In case of figure 2A the pancreas size highlighted in KC mice is different from what is shown in panel 1D of figure1. Is there any reason for this or it is reflection of the way the picture is taken thus hiding the rest of the pancreas? Secondly, since the tumor in the KTC mice is bigger than the KC mice this may predict more metastasis observed in KTC mice.

Authors
Indeed, the difference in size of the pancreas between Fig 2A and Fig 1D is a reflection of the way the picture is taken. To take pancreas pictures inside the abdomen, we preserve the orientation of the tissue in order to avoid altering the liver (principal site of PDAC metastasis), which is located in close proximity to the pancreas (please see pictures in Fig 2A). To take pancreas pictures after tissue collection, the excised pancreas is unrolled before the photo acquisition in order to show both the head and the tail of the pancreas. This approach is discussed in the revised manuscript (Materials and Methods/page30/paragraph1).

We do agree with the Reviewer that the difference in tumor sizes between *KC* and *KTC* mice might affect the interpretation of the metastasis data. In efforts to address this issue, we conducted further microscopic examination, which showed complete absence of micrometastases in the liver or lung of *KC* mice. Microscopic examination of pancreas also showed that *KTC* tumors displayed uniformly poorly differentiated architecture that occupied the entire pancreas, whereas age-matched *KC* mice developed PanINs that were confined within large areas of normal pancreas exhibiting well-organized acinar architecture (Figs 1E, 2B, EV1K, EV2A), which is consistent the difference in the metastatic phenotypes observed between *KC* and *KTC* mice at the same age. These observations are discussed in the revised manuscript (Results/page10-11/from paragraph1).

Reviewer 3
In figure 2D loading control has to be incorporated to validate the findings.

Authors
We included b-Actin as a loading control (Fig 2A).

Reviewer 3
In figure 3 panel (D and E), right panel looks like representation of densitometric analysis of the left panel or if it represents independent experiment has to be made clear in the figure legend. Secondly, it would be good to show only one panel here and move the other panel in the supplementary figures.

Authors
Right panel and left panel in Figs 3D and 3E (now Figs 3F, 3G, EV3G and E3VH) represent similar but independent ChIP experiments using the same chromatin samples. In Fig 3D, the ChIP reaction was analyzed by agarose gel following PCR. In Fig 3D, the ChIP reaction was analyzed directly by quantitative PCR (qPCR). For clarity, we indicated this information in the "Materials and Methods" section (page32/paragraph2). In addition, we moved the right panels to the "Extended View" section (Figs EV3G and E3VH), as per Reviewer's suggestion.

Reviewer 3
For figure 4G it would be important to show the IHC panel for p16 protein also along with E-cadherin and vimentin.

Authors
We performed this IHC experiment, and the results confirmed our qRT-PCR data (Figs 4F and 4G). We included this new result in the revised manuscript (Result/page15/paragraph2).

Reviewer 3
In case of Figure 5 expression of cdh1, vimentin and p16 has been shown at transcript level however, it would be a good to include IHC/western blot assay for the same to show expression at protein levels to keep it consistent throughout the manuscript.

Authors

We performed these IHC experiments, and the results confirmed the results obtained at the transcript levels (Figs 5C-E and EV5D). These new results are discussed in the manuscript (Result/page16/paragraph1).

Reviewer 3
In case of figure 6 stable cell lines were generated expressing TGIF1 and mutant of TGIF1 and expression of same are shown in panel C however, the respective empty vehicle panel and loading control is not shown for this panel.

Authors
As suggested by the Reviewer, we included a loading control (b-Actin) in Fig.6C (Now Fig 7C). The expression of TGIF1 and TGIF1.2TD in Fig.6C was detected using anti-Flag antibody. To simplify the presentation of data (loading TGIF1 and TGIF.2TD together), we omitted the empty vector lanes because this empty vector was already used in Fig.6A, and the anti-Flag antibody detected no band. We never detected a band with the empty vector in a huge number of experiments performed in our lab, many of them were already published in several papers.

Reviewer 3
The results state that Twist1 expression depends only on the level of TGIF1 phosphorylation and not on quantity of TGIF1 protein. Thus, the model on Fig. 7 would better represent the results if the amount of TGIF1 "rectangles" on the left part of model will be the same as on the right part but only (e.g. 1 of 3) will be marked as phosphorylated.

Authors
We modified the model (now Fig 7E), as per Reviewer's suggestion.

Reviewer 3
Major concerns:

Reviewer 3
For figure 2B lymph node panel describing metastasis in KTC mice should be validated by staining with some markers of invasion.

Authors
We performed an immunofluorescence experiment using an antibody to Vimentin, a prominent marker of invasion (Fig 2B). The result, which confirmed the presence of invasive cells in lymph node of *KTC* mice, is discussed in the revised manuscript (Results/page10/paragraph2).

Reviewer 3
In figure 3A Input for the CoIP experiment is not presented and for panel C densitometric analysis should be provided. Reciprocal CoIP for the same is missing to further validate the data. For figure 2B in case of TGIF KO panel because of the absence of TGIF in input as it is knock out mice therefore, it is obvious to not find interaction between Twist1 and TGIF1. The best way to prove this point will be to use an inducible system and show that interaction persist in the absence of inducer and diminish in presence of inducer.

Authors
-We included input for the CoIP in Fig 3A. We also conducted densitometric analysis for Fig 3C (now Fig 3E), and the result was presented as a ratio of Twist1/TGIF1. Reciprocal coimmunoprecipitation for TGIF1 and Twist1 was also performed (Fig 3A).

-For Fig 2B, we wanted to present interaction data with endogenous TGIF1 and Twist1 in the pancreatic tissue. We used $Tgif1^{KO}$ pancreatic extracts in order to attest to the specificity of the endogenous TGIF1-Twist1 interaction. The inducible system would likely rely on overexpressed proteins, and we already presented the data with an overexpression system in Fig EV3A. We believe that the experiment shown in Fig EV3A closely resemble the inducible system suggested by the Reviewer, in that it allows analysis of the interaction in cells transfected with Twist1 in complete absence or presence of TGIF1, similar to what can be achieved with the inducible system. We observed an interaction only in the presence of TGIF1 (Fig EV3A). These results are discussed in the revised manuscript (Results/page12/paragraph1).

Reviewer 3
CoIP or protein interaction assays should be performed to show the effect on the association of twist1 with TGIF1 when its phosphorylated. Further, effect of TGIF1 phosphorylation on the expression of TWIST1 target genes should be shown to underscore the importance of TGIF1 phosphorylation function in disrupting its anti-tumor activity. Along the same line, it should be checked that whether specific inhibitor for phosphorylation can revert the observations.

Authors
As suggested by the Reviewer, we used the BxPC3 cell line, the only human PDAC cell line reported to harbor wild-type *KRAS*, to the best of our knowledge. We found that TGIF1 is more phosphorylated in MiaPaca2 cells (which harbors $KRAS^{G12C}$) than in BxPC3 cells (Fig EV6A), consistent with our model. This result is discussed in the revised manuscript (Results/page16/paragraph2).

Unluckily, we found that expression of Twist1 in BxPC3 cells is low, hampering any attempt to use this human PDAC cell line in coimmunoprecipitation assays to detect the association between endogenous pTGIF1 and Twist1. Further, the transfection efficiency did not allow us to conduct coimmunoprecipitation with overexpressed proteins. As an alternative approach to overcome this limitation, we sought to use the mammalian two-hybrid system (described in our previous publications, *Cell Reports* 4:530-541, 2013 and *Cancer Cell* 27:547-560, 2015), which proved to be a more sensitive method to analyze the interaction of TGIF1 with Twist1 in BxPC3 cells co-transfected with $Kras^{G12D}$, used to trigger TGIF1 phosphorylation as in human PDAC cell lines with oncogenic *KRAS* used in our study. As anticipated from our coimmunoprecipitation experiments, we detected a specific and robust interaction between TGIF1 and Twist1 in cells cotransfected with empty vector (Fig EV6C). More importantly, that interaction was decreased when $Kras^{G12D}$ was cotransfected (Fig EV6C). Under these experimental conditions, TGIF1.2TA (which specifically inhibits TGIF1 phosphorylation) interacted more strongly with Twist1, and this interaction was not affected by $Kras^{G12D}$ (Fig EV6C). This result is discussed in the revised manuscript (Results/page17/paragraph2).

-In attempts to confirm these data, we found that $Kras^{G12D}$ expression blunted the ability of wild-type TGIF1 to repress Twist1 expression (Fig 6F), as assessed by our gene porter assay using TGT^{Luc} (luciferase driven by the Twist1 promoter). In marked contrast, $Kras^{G12D}$ did not interfere with the ability of TGIF1.2TA to repress Twist1 expression (Fig 6F). This result is discussed in the revised manuscript (Results/page17/paragraph3).

-In attempts to investigate the effects of TGIF1 phosphorylation on the expression of TWIST1 target genes, we were not able to obtain BxPC3 cells stably expressing the phosphorylation-defective mutant TGIF1.2TA, perhaps because of its increased propensity to inhibit cell proliferation. To circumvent this limitation again, we decided to perform gene reporter assays using luciferase under the control of the *CDH1* ($CDH1^{Luc}$) or *p16INK4A* ($p16^{Luc}$) promoters. We found that TGIF1.2TA was more effective than the wild-type counterpart at suppressing Twist1-mediated repression of $CDH1^{Luc}$ and $p16^{Luc}$ (Figs 6G and 6H). More importantly, expression of $Kras^{G12D}$ blunted the effect of wild-type TGIF1, but failed to do so when TGIF1.2TA was contransfected (Figs 6G and 6H). These results are described in the revised manuscript (Results/page17/paragraph3).

Reviewer 3
It would be of due importance to go for RNA-seq/micro-array in TGIF KO/KC/KTC conditions to identify the genes and pathways that gets modulated. This experiment can provide insight into some new targets and pathways related to EMT or TGF-beta/smad signaling or may be Wnt signaling considering TGIF1 has a role to play in Wnt-signaling and will strengthen the impact of this study.

Author
Unluckily, we do not have enough adequate samples to conduct those experiments. Generating new $Tgif1^{KO}$, *KC*, *KTC*, and *KTWC* mice that develop PDAC (or no) will require a minimum of 15 months since we keep all our colonies with individual transgenes separated in order to facilitate other undergoing studies and also avoid unnecessary pain to mice which are projected to develop PDAC. Although we were not able to provide these data, the genetic and epigenetic alterations in human PDAC have already been extensively characterized in many integrative genome and transcriptome studies performed by The Cancer Genome Atlas (TCGA), International Cancer

Genome Consortium (ICGC), and other consortiums or independent laboratories. In contrast, the roles of posttranslational modifications in PDAC remain poorly investigated. The fact that TGIF1's function is mainly regulated by phosphorylation provided us with a unique opportunity to investigate whether this previously uncharacterized posttranslational event could impact TGIF1's functional interactions with other proteins that might play a determinant role in PDAC. To probe this possibility, we choose to perform an integrative protein-protein interaction study by means of the yeast two-hybrid system using a general cDNA library that encompasses the vast majority of genes expressed in mammalian cells. To be focused, we decided to characterize the interaction of TGIF1 with one of the top candidates, the pro-malignant transcription factor Twist1, keeping in mind the stringent criteria that such choice will endowed us with the capability to use Twist1 conditional mice in genetic approaches to provide compelling evidence as to whether TGIF1 exerts its tumor suppressor function in PDAC by a molecular mechanism involving Twist1, despite being expected to be excessively time-consuming. With that being said, we totally agree with the Reviewer that it would be more informative to use other integrative approaches, such as RNA sequencing, to identify other genes and pathways that play causative roles in PDAC, and we are deeply grateful to the Reviewer for this important critique and all other constructive critiques as well. We are definitely planning to generate again *Tgif1*^{KO}, *KC*, *KTC*, and *KTWC* mice deleted of *p16ink4a* or *Cdh1* to conduct RNA sequencing, ChIP-sequencing, proteomic, and lipodomic experiments. It is our sincere hope to be able to provide the PDAC field in future studies with additional observations that could be exploited both in terms of understanding the mechanistic underpinnings of PDAC, and in terms of translational opportunities. We discussed this possibility in the revised manuscript (Discussion/page22/paragraph1).

In closing, we would like to express our profound gratitude to this Reviewer as well as to the other Reviewers for helping us to build up a compelling story about the new role of TGIF1 as a tumor suppression in PDAC, a highly aggressive and lethal malignancy.

2nd Editorial Decision 27th Mar 2019

Thank you for submitting your revised manuscript for consideration by The EMBO Journal as well as giving us additional information regarding the data acquisition. As mentioned, your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication, pending minor revisions.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues stated by referee #3, as well as formatting changes as outlined below, which need to be adjusted at re-submission.

As to referee #3's remaining points, please revisit the discussion and integration of recent literature and findings and see of you would be able to the add complementary qPCR and invasion assay data requested or alternatively relativise your statements and introduce caveats in the manuscript where appropriate.

REFEREE REPORTS:

Referee #1:

The authors have addressed my previous critique and concerns to satisfaction and I find the work suitable for publication in the Journal.

Referee #2:

In my opinion the authors have adequately and sufficiently responded to the reviewers' comments. In particular, they have included additional important experimental evidence to support the

conclusions, they have improved the quality of the presentation of the results, expanded on the statistical significance of some of the experiments, notably on the expression of Tgif1 and Twist in patient samples, and they have revised and improved the manuscript accordingly.
The manuscript reports a very important new regulatory mechanism in the development and progression of PDAC with high relevance for the basic understanding of malignant PDAC and general cancer progression and for the further development of therapeutic approaches in a clinical setting.

Referee #3:

General Summary: This manuscript adds new and interesting findings that will strengthen the understanding of novel role of TGIF1 in molecular events leading to progression of PDAC with KRAS mutation.

Comments for the revised manuscript:
The authors have convincingly addressed most of the points that were raised in previous version of the manuscript. The manuscript in current form can be considered for publication in agreement with justifications of other reviewer's comments. There are some minor concerns that authors failed to describe but these points can be discussed in the revised manuscript like; role of TGIF1 as an oncoprotein in case of breast cancer. There is evidence of role of TGIF1 in promoting invasion at least in case of TNBC (Yeon Jin Kwon etal 2016; Oncotarget) where authors have described that TGIF knockdown inhibits invasion though, it cannot be ruled out that this observation was not seen in context of KRAS. In view of the available literature, authors can describe that TGIF1 function as a tumor suppressor in a context dependent manner and this observation is specific to PDAC and can include such references in their manuscript.
Considering the fact that authors cannot perform RNA-seq or ChIP-seq they can at least check some of the genes that regulates wnt signaling and invasion in their cell line system by qPCR to further strengthen their study (Addressing this point is based on editor discretion).

Although vimentin is known to be a marker of invasion but is shown to be upregulated in the absence of TGIF1 therefore, some other invasion assays like Boyden chamber can be done in In vitro system that can substantiate these findings (The editor can take the final decision whether to address this or not).

2nd Revision - authors' response 11th Apr 2019

Responses to the Reviewers

Referee #1
The authors have addressed my previous critique and concerns to satisfaction and I find the work suitable for publication in the Journal.

Referee #2
In my opinion, the authors have adequately and sufficiently responded to the reviewers' comments. In particular, they have included additional important experimental evidence to support the conclusions, they have improved the quality of the presentation of the results, expanded on the statistical significance of some of the experiments, notably on the expression of Tgif1 and Twist in patient samples, and they have revised and improved the manuscript accordingly. The manuscript reports a very important new regulatory mechanism in the development and progression of PDAC with high relevance for the basic understanding of malignant PDAC and general cancer progression and for the further development of therapeutic approaches in a clinical setting.

Referee #3:
General Summary: This manuscript adds new and interesting findings that will strengthen the understanding of novel role of TGIF1 in molecular events leading to progression of PDAC with KRAS mutation.

Comments for the revised manuscript

Reviewer3
The authors have convincingly addressed most of the points that were raised in previous version of the manuscript. The manuscript in current form can be considered for publication in agreement with justifications of other reviewer's comments. There are some minor concerns that authors failed to describe but these points can be discussed in the revised manuscript like; role of TGIF1 as an oncoprotein in case of breast cancer. There is evidence of role of TGIF1 in promoting invasion at least in case of TNBC (Yeon Jin Kwon et al 2016; Oncotarget) where authors have described that TGIF knockdown inhibits invasion though, it cannot be ruled out that this observation was not seen in context of KRAS. In view of the available literature, authors can describe that TGIF1 function as a tumor suppressor in a context dependent manner and this observation is specific to PDAC and can include such references in their manuscript.

Authors
As suggested by the Reviewer, we discussed in the manuscript that TGIF1 might function as a tumor suppressor in a context dependent manner and included the reference kindly pointed out by the Reviewer (Discussion/page-25/paragraph-2).

Reviewer3
Considering the fact that authors cannot perform RNA-seq or ChIP-seq they can at least check some of the genes that regulates wnt signaling and invasion in their cell line system by qPCR to further strengthen their study (Addressing this point is based on editor discretion).

Authors
As suggested by the Reviewer, we performed qRT-PCR to analyze the expression of two Wnt target genes, Axin2and Lgr5. The result revealed a marked increase in the expression Axin2and Lrg5in KC mice, but this effect was not significantly changed in *KTC* mice (Appendix Fig S1D), suggesting that KrasG12Dexpression might render Wnt signaling independent of TGIF1. We discussed this new result in the revised version of the manuscript (Results/page-11/paragraph-2)

Reviewer
Although vimentin is known to be a marker of invasion but is shown to be upregulated in the absence of TGIF1 therefore, some other invasion assays like Boyden chamber can be done in In vitro system that can substantiate these findings (The editor can take the final decision whether to address this or not).

Authors
We present data in the manuscript showing that Tgif1ablation in KTC mice culminatedin a marked increase in Vimentin expression, and this effect was blunted upon ablation of Twist1, providing strong evidence that TGIF1 regulates Vimentin expression through its ability to antagonize Twist1 (Figs2B, 4C, 4E, 4G and5D). We also found that *Tgif1*ablation in KTC mice promoted PDAC metastasis to several organs associated with human PDAC (e.g., liver, lung, lymph node), and this effect was again blunted by the ablation of Twist1(Figs 2B, 5BandS4D).Finally, we performed tracing experiments in live animals to confirm that Tgif1ablation can promote metastasis(Fig2B). Collectively, these experiments provide evidence supporting the involvement of TGIF1 in PDAC metastasis. However, since TGIF1 also affects cell proliferation, we do not exclude the possibility that TGIF1 might indirectly promotes PDAC metastasis, perhaps without affecting the invasive behavior of PDAC cells. We believe that this issue raised by the Reviewer is important, but addressing it will require extensive studies to identify PDAC cell lines in which TGIF1 expression does not affect cell proliferation in order to firmly dissociate the effects ofTGIF1 on cell proliferation and cell invasion. At present, expression of TGIF1 inhibits the proliferation of all PDAC cell lines analyzed (5 different cell lines). We discussed this issue in the revised version of the manuscript (Discussion/page-23/paragraph-1)

3rd Editorial Decision 23rd Apr 19

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Azeddine Atfi
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-101067

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?	For every experiment, sample size was determined empirically (preliminary experiments were performed) to ensure that the desired statistical power could be achieved. The values are expressed as mean ± SEM. The error bars (SEM shown for all results were derived from biological replicates, not technical replicates. Significant differences between two groups were evaluated
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least 6 mice per group were used in all experiments involving mice.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion criteria was used. All mice irrespective of gender, age, weight, background or health stautus were included.
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.	No treatment was done.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
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.	Scoring the immunohistochemistry or immunfluorescence data was done in a blinded manner (blinding of the investigator).
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical analysis is included in all figures
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes. Materials and Methods, Statistical Analysis.

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).	Details of antibodies can be found in Material and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of all cell lines was indicated in Materials and Methods. Cells are free of mycoplasma.

* 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.	All details provided in material and methods. Source of animals and housing and husbandry conditions are provided.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi Medical Center.
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.	All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi Medical Center. We strictly follow the American Veterinary Medical Association Panel guidlines.

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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