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PTPN3 Acts as a Tumor Suppressor and Boosts TGF-b Signaling Independent of its Phosphatase Activity

Bo Yuan, Jinquan Liu, Jin Cao, Yi Yu, Hanchenxi Zhang, Fei Wang, Yezhang Zhu, Mu Xiao, Sisi Liu, Youqiong Ye, Le Ma, Dewei Xu, Ningyi Xu, Yi Li, Bin Zhao, Pinglong Xu, Jianping Jin, Jianming Xu, Xi Chen, Li Shen, Xia Lin, Xin-Hua Feng

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Revision received:	14th Mar 2019
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Editor: Deniz Senyilmaz Tiebe

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 13th Jul 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. As you can see, all referees express interest in the proposed mechanism by which PTPN3 regulates TGF-β signaling. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here. Most importantly, referee #3 asks you to elaborate on the cancer relevance of the findings and to demonstrate the interaction between PTPN3 and TβRI at the endogenous level. Moreover, referees 1 and 2 raise some concerns about the cell and mouse models used in the study.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS:

Referee #1:

In this study, Yuan et al. investigated the role of PTPN3 in regulating TGF-β signaling and TGF-β-mediated hepatocellular carcinoma suppression. Mechanistically, PTPN3 inhibits ubiquitination-dependent degradation of TβRI by antagonizing Smurf2 in a phosphatase-independent manner, thus enhancing TGF-β-mediated transcriptional responses and growth inhibitory effects on HCC. Furthermore, the authors revealed that a PTPN3 mutant at L232R, a frequent mutation found in intrahepatic cholangiocarcinoma (ICC), has the capacity to disable the function of PTPN3 on enhancing TGF-β signaling and TGF-β-mediated HCC suppression. PTPN3 has been reported to repress a variety of human cancers, this paper further interpret the mechanisms underlying how

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PTPN3 affects tumor progression in liver cancer. Overall, the data are well organized and most of the results for the major part are supportive of the paper's conclusions. While the findings are interesting and potentially important, the following concerns need to be addressed.

1. The authors mainly used HaCaT cells and A549 cells to show that PTPN3 is able to promote TGF-β-induced transcriptional responses in Figure 1 and S1. To make the same conclusion in HCC cells, which would support the functions of PTPN3 in HCC progression in following parts of the paper, the authors should repeat the PTPN3-knockdown/overexpression and rescue experiments in at least one HCC cell line (such as Huh7 cells).

2. In Figure 1g, the western blot data showed that TGF-β could also induced PTPN3 protein levels. The authors should confirm if PTPN3 expression is stimulated by TGF-β, which could be served as a positive-feedback loop between TGF-β signaling and PTPN3 in the process.

3. It has been reported that PTPN3 (L232R), which was frequently detected in intrahepatic cholangiocarcinoma (ICC) samples, were found to be gain-of-function mutations. Moreover, PTPN3 (L232R) expression in ICC cell lines increases cell proliferation, colony formation and migration (Gao et al., Gastroenterology 2014; 146:1397-1407). Thus, this citation in the paper is not appropriate to support the notion that PTPN3 appears to act as a tumor suppressor in cancer progression in the Introduction Section. It seems that the author should discuss and be carefully to make the conclusion that PTPN3 mutant with the L232R substitution that occurs frequently in ICC has completely lost its ability to enhance TGF-β signaling.

4. The authors revealed that depletion of PTPN3 further promoted tumor formation (Figure 6c-e). To further convince that PTPN3 (L232R) exhibited its blocking effect on TGF-β-mediated growth responses, the authors should performed in vivo experiment to detect tumor growth in HepG2 cells expressing WT-PTPN3, PTPN3 (D811A) or PTPN3 (L232R) mutant.

Referee #2:

In the present study, the authors identified protein tyrosine phosphatase non-receptor 3 (PTPN3) as a novel positive regulator of TGF-b signaling. Unexpectedly, PTPN3 enhances TGF-b signaling independently of its phosphatase activity, but through interfering with the negative regulation mediated by Smurf2. Importantly, L232R mutant of PTPN3, which is frequently found in intrahepatic cholangiocarcinoma, loses the activity to augment TGF-b signaling but retains the phosphatase activity, suggesting that dysregulation of TGF-b signaling is more important in cholangiocarcinogenesis. Moreover, they demonstrated a tumor suppressor function of PTPN3 in liver cancer using mouse xenograft model. Overall, biochemical experiments are well performed and results support the authors' conclusions. However, I have several concerns.

Major concerns
1) PTPN3(L232R) obviously suppresses TGF-b signaling (Figure 5e and f, Figure 6a and b). Consistently, it enhanced Smurf2-induced ubiquitination of TbRI (Figure 5d) and downregulated TbRI (Figure 5a). It appears that PTPN3(L232R) not only lost activity to enhance TGF-b signaling but also acquired a novel function to suppress TGF-b signaling. This finding should be, at least, commented and discussed, although the authors appear to avoid mentioning it.

2) As presented in Synopsis, Smurf2-TbRI interaction and ubiquitination of TbRI by Smurf2 were previously shown to be dependent on Smad7 (Kavsak et al., 2000). In the present study, however, the authors did not examine how PTPN3 affects Smad7 function in recruiting Smurf2 to TbRI. Does PTPN3 inhibit the Smad7-TbRI interaction or Smad7-Smurf2 interaction? At least, this point should be commented.

Minor concerns
1) PTPN3(L232R) binds to TbRI, similarly to PTPN3 wt and PTPN3(D811A), but fails to block Smurf2-TbRI interaction. This is unexpected and not easy to understand. Discuss the underlying mechanism.

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2) Supplementary Figure 2: The authors confirmed that PTPN3(L232R) mutant retained phosphatase activity. This is an important finding and can be mentioned in page 9, where PTPN3 is first described in the main text.

3) The authors used HeG2 cells in mouse xenograft assays. However, ATCC data sheet says that HepG2 is not tumorigenic in immunocompromised mice. Are the cells really HepG2 cells? https://www.atcc.org/products/all/HB-8065.aspx#characteristics

4) Show TbRI blot in Figure 5e. Readers would like to know why Smad2/3 phosphorylation was totally inhibited by PTPN3(L232R).

5) Page 17, Materials and Methods: Delete the section of Immunofluorescence.

6) Figure 1c-f: The authors may have mixed up data for p21 and PAI-1. siRNA-1 completely inhibited TGF-b-induced up-regulation of p21 at 8 h after stimulation in Figure 1c while only partially in Figure 1e. Similarly, siRNA-1 only partially inhibited TGF-b-induced up-regulation of PAI-1 at 8 h after stimulation in Figure 1d while completely in Figure 1f.

7) Figure 5e: Explain (-) and (+) in the legend.

8) Figure 6b and others: Quantification of the results in clonogenic assays (as described in Materials and Methods, page 18) should be presented.

9) Figure 6f: Were cells treated with TGF-b? This is not clear.

10) Page 4, line 8 from the bottom: liver progression >> liver cancer progression?

Referee #3:

Yuan and co-authors present a set of experimental approaches that link the action of the PTPN3 tyrosine phosphatase to regulation of signaling by the receptor kinase for the cytokine transforming growth factor beta (TGFbeta). Overall, the main conclusions of this paper are interesting with respect to their importance in the regulation of TGFbeta signal transduction. The approaches are well established in the field and in the laboratory of Dr. X-H. Feng and involve primarily biochemical interaction assays in transfected cell models. Similar to PTPN3, the Feng and other labs have previously established numerous independent regulators of TGF-beta signaling. The novelty of the report on PTPN3 is that this protein is an established tyrosine phosphatase, which acts as a TGF-beta receptor regulator in the absence of its catalytic activity. This interesting finding and the identification of a genetic mutation in PTPN3 (L232R) that does not affect phosphatase activity, yet it disrupts the regulation of the TGF-beta receptor provide exciting ground based on which the authors may build a deeper analysis of the mechanism by which PTPN3 regulates the TGF-beta receptor. In view of the current understanding of regulation of TGF-beta signaling, this appears to be a necessary additional set of experiments that will explain deeper the function of PTPN3 as a TGF-beta regulator.

1. Essentially all biochemical experiments use transfected proteins. Is it possible to demonstrate the endogenous complex between PTPTN3 and TbetaRI?
2. Does shRNA against the endogenous PTPN3 intefere with the interaction between Smurf2 and the TbetaRI?
3. Smurf2 is thought to bind the TGFbeta receptor via the adaptor protein Smad7. Does Smad7 make a complex with PTPN3 or is the interaction between PTPN3 and the receptor direct?
4. Which part of the TBetaRI is involved in the interaction with PTPN3?
5. How does the FERM domain and specifically Leu232 regulate the TGF-beta receptor? This requires further analysis? Does this interaction take place on the plasma membrane or on endosomal membranes?
6. Since L232R occurs in ICC, is it possible to analyse TGF-beta signaling in samples from such patients by immunohistochemistry or RNA-based methods? Is TGF-beta cytostatic or pro-tumorigenic in cholangiocytes?
7. Since PTPN3 regulates TGF-beta receptor signaling, all responses to TGF-beta are affected by

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PTPN3. This is necessary to demonstrate beyond the 3 genes examined (p21, myc, PAI-1) and cell proliferation. Does PTPTN3 affect all gene responses to TGF-beta?
8. Is the action of PTPN3 specific for epithelial cells or does it also regulate TGF-beta signaling in fibroblasts, lymphocytes or stem cells?

I also enlist some technical and stylistic comments:

9. The methods will be more complete if the analysis of TCGA expression data and Kaplan-Meier analysis is explained.
10. The acknowledgments describe a series of human liver cancer cell lines, which are not described in the methods or results. The acknowledgements can be amended accordingly.
11. The discussion has large sections that simply repeat the results and reiterate many times the same points. The discussion on TGF-beta being tumor suppressive or pro-tumorigenic and PTPN3 having similarly dual roles is confusing. I thought that the paper tries to make a case that PTPN2 acts in a tumor suppressive manner. This part of the discussion can be clarified better or omitted.

1st Revision - authors' response 25th Jan 2019

Point-to-point reply to the reviewers' comments:

We thank the reviewers for their positive review and constructive comments of our manuscript. We carefully followed the reviewers' instructions and conducted the suggested experiments and also made extra efforts to carry additional experiments. In re-writing this manuscript, we have incorporated the new data, made corrections and re-written description where had not been clear. We believe the revised manuscript has answered all the questions raised by the reviewers and gone beyond. Thus, this revised manuscript has significantly been strengthened.

Please note that in the revised manuscript the following are new data:

- Fig 1H-K;
  Fig 1C-G are new data to replace corresponding old data.
  Fig EV1B, C, F, J, K & L (related to Fig 1);
- Fig 4D and E;
  Fig 4B are new data to replace corresponding old data.
  Fig EV3A-E (related to Fig 4);
- Fig 5A and E;
  Fig EV4A-D (related to Fig 5);
- Fig 7A-C;
- Fig EV5D-F (related to Fig 6 & 7).

In the following pages, we have a point-to-point reply to the editor's summary and each reviewer's comments.

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Referee #1:

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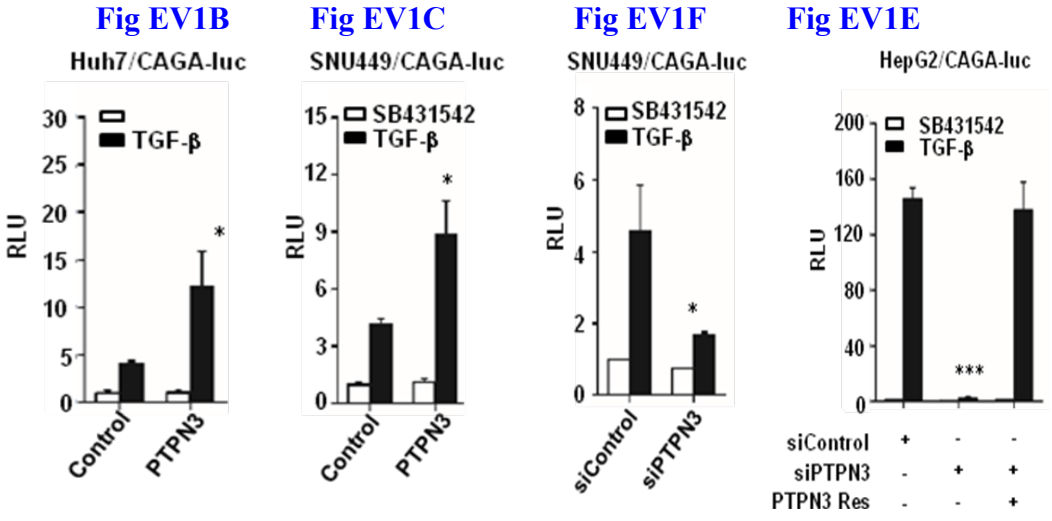
1. The authors mainly used HaCaT cells and A549 cells to show that PTPN3 is able to promote TGF-β-induced transcriptional responses in Figure 1 and S1. To make the same conclusion in HCC cells, which would support the functions of PTPN3 in HCC progression in following parts of the paper, the authors should repeat the PTPN3-knockdown/overexpression and rescue experiments in at least one HCC cell line (such as Huh7 cells).

Response: We agree with the Reviewer and thus determined the effect of altered PTPN3 expression on TGF-β transcriptional responses in hepatocellular carcinoma Huh7 cells and SNU449 cells. In agreement with results in Fig 1 and Fig EV1, overexpression of PTPN3 enhanced TGF-β-induced CAGA-luc responses in Huh7 (Fig EV1B) and SNU449 cells (Fig EV1C), whereas knockdown of PTPN3 expression significantly attenuated the CAGA-luc response in SNU449 (Fig EV1F) and HepG2 cells (Fig EV1G). Moreover, the effect of PTPN3 siRNA could be rescued by expression of an RNAi-resistant variant of PTPN3 (Fig EV1E and G).



2. In Figure 1g, the western blot data showed that TGF-β could also induced PTPN3 protein levels. The authors should confirm if PTPN3 expression is stimulated by TGF-β,

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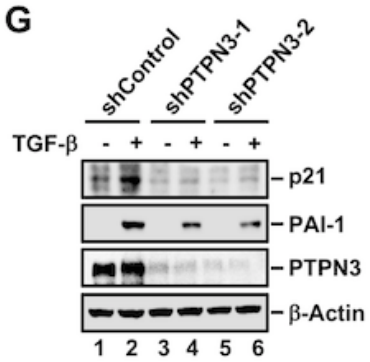
which could be served as a positive-feedback loop between TGF-β signaling and PTPN3 in the process.

Response: We have redone and replaced the data in Fig 1G. The old blot might have not been evenly transferred in Western blotting. We also did a time course to examine if TGF-β induces PTPN3 protein levels. As shown below, TGF-β does not affect the PTPN3 expression level in HaCaT cells.

Fig 1G **Below data for review only**



[Figure for reviewers removed]

3. It has been reported that PTPN3 (L232R), which was frequently detected in intrahepatic cholangiocarcinoma (ICC) samples, were found to be gain-of-function mutations. Moreover, PTPN3 (L232R) expression in ICC cell lines increases cell proliferation, colony formation and migration (Gao et al., Gastroenterology 2014; 146:1397-1407). Thus, this citation in the paper is not appropriate to support the notion that PTPN3 appears to act as a tumor suppressor in cancer progression in the Introduction Section. It seems that the author should discuss and be carefully to make the conclusion that PTPN3 mutant with the L232R substitution that occurs frequently in ICC has completely lost its ability to enhance TGF-β signaling.

Response: Thank the reviewer for his/her kind suggestions. Based on our own data, we believe that PTPN3 is a tumor suppressor through its enhancement of TGF-β receptor stabilization. The L232R mutant found in ICC apparently lost such ability. Indeed, the study reported by Gao *et al* emphasized the gain-of-function toward oncogenic potential. We think these two aspects are not necessarily contradictory. For example, mutations in the TP53 such as R372 also cause the loss of tumor suppressor activity and gain of oncogenicity. Therefore, we have revised the Introduction/Discussion sections.

4. The authors revealed that depletion of PTPN3 further promoted tumor formation (Figure 6c-e). To further convince that PTPN3 (L232R) exhibited its blocking effect on TGF-β-mediated growth responses, the authors should performed in vivo experiment to detect tumor growth in HepG2 cells expressing WT-PTPN3, PTPN3 (D811A) or PTPN3 (L232R) mutant.

Response: We appreciate the reviewer's kind constructive suggestion. We first performed *in vivo* tumor formation assay in HepG2 cells stably expressing WT-PTPN3 , PTPN3 (D811A) or PTPN3 (L232R) and parental HepG2 cells. As shown

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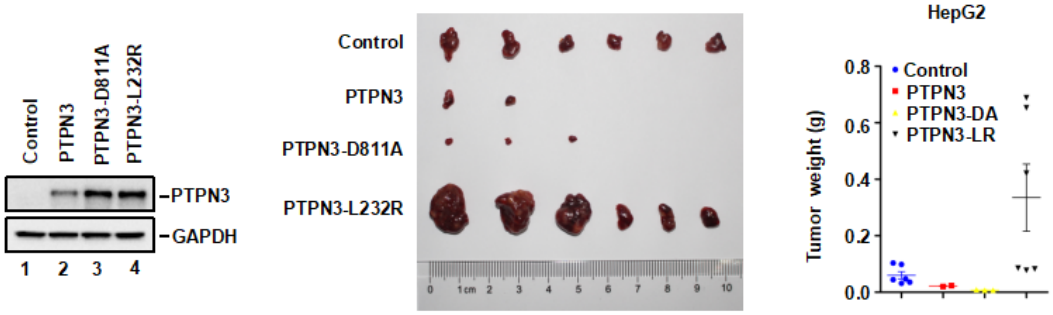
below (Fig EV5D-F, HepG2 cells) and consistent with the result in Fig 7A-F (Huh7 cells), both WT-PTPN3 and its phosphatase-dead mutant PTPN3 (D811A) inhibited tumor formation, whereas PTPN3 (L232R) lost its tumor suppressing activity.

Fig EV5D **Fig EV5E** **Fig EV5F**



Referee #2:

In the present study, the authors identified protein tyrosine phosphatase non-receptor 3 (PTPN3) as a novel positive regulator of TGF-b signaling. Unexpectedly, PTPN3 enhances TGF-b signaling independently of its phosphatase activity, but through interfering with the negative regulation mediated by Smurf2. Importantly, L232R mutant of PTPN3, which is frequently found in intrahepatic cholangiocarcinoma, loses the activity to augment TGF-b signaling but retains the phosphatase activity, suggesting that dysregulation of TGF-b signaling is more important in cholangiocarcinogenesis. Moreover, they demonstrated a tumor suppressor function of PTPN3 in liver cancer using mouse xenograft model. Overall, biochemical experiments are well performed and results support the authors' conclusions. However, I have several concerns.

Major concerns
1) PTPN3(L232R) obviously suppresses TGF-b signaling (Figure 5e and f, Figure 6a and b). Consistently, it enhanced Smurf2-induced ubiquitination of TbRI (Figure 5d) and downregulated TbRI (Figure 5a). It appears that PTPN3(L232R) not only lost activity to enhance TGF-b signaling but also acquired a novel function to suppress TGF-b signaling. This finding should be, at least, commented and discussed, although the authors appear to avoid mentioning it.

2) As presented in Synopsis, Smurf2-TbRI interaction and ubiquitination of TbRI by Smurf2 were previously shown to be dependent on Smad7 (Kavsak et al., 2000). In the present study, however, the authors did not examine how PTPN3 affects Smad7 function in recruiting Smurf2 to TbRI. Does PTPN3 inhibit the Smad7-TbRI interaction or Smad7-Smurf2 interaction? At least, this point should be commented.

Minor concerns
1) PTPN3(L232R) binds to TbRI, similarly to PTPN3 wt and PTPN3(D811A), but fails to block Smurf2-TbRI interaction. This is unexpected and not easy to understand. Discuss the underlying mechanism.

Response： These three raised points of the reviewer are related, so we reply them together in the following. Though not asked by the reviewer, we carried out additional experiments to address the mechanism underlying the differences of PTPN3(L232R) vs. PTPN3 wt on TβRI regulation. The key point

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is that the binding of PTPN3(L232R) to TβRI differs from that of PTPN3 wt.

1. We found that deletion of the GS region of TβRI disabled its interaction with PTPN3 (Fig EV3B). The GS region is where Smad7 binds to and recruit Smurf2 to degrade TβRI. The observation that the GS mutant of TβRI failed to interact with PTPN3 fits our working model where PTPN3 prevents TβRI degradation. Interestingly, the L232R mutant binds equally well to wildtype TβRI and the GS deletion mutant of TβRI (Fig EV4C), suggesting that the L232R mutant bind to TβRI in a different way from PTPN3 wildtype.

Fig EV3B

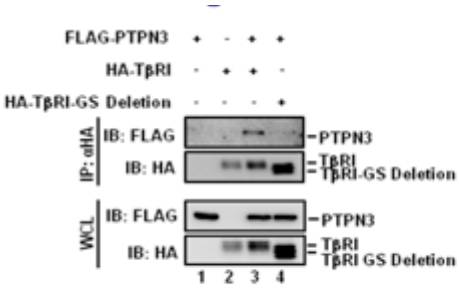
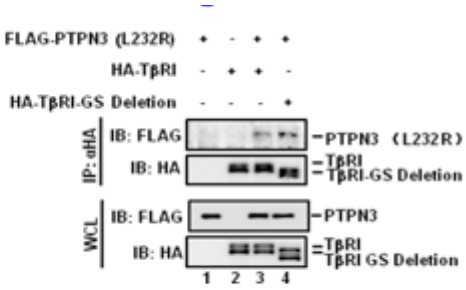


Fig EV4C



2. We examined how PTPN3 affects Smad7's function in recruiting Smurf2 to TβRI. We carried two IP experiments: one is to examine whether PTPN3 affects the Smad7 binding to TβRI; the other is to determine whether PTPN3 affects the Smad7-Smurf2 interaction. As shown below, ectopic expression of PTPN3 (wildtype or phosphatase-dead D811A) abolished the Smad7-TβRI interaction, while the L232R mutant had no effect on the interaction (Fig 5E). PTPN3 did not affect the Smurf2-Smad7 interaction *per se* (Fig EV3E)

Fig 5E

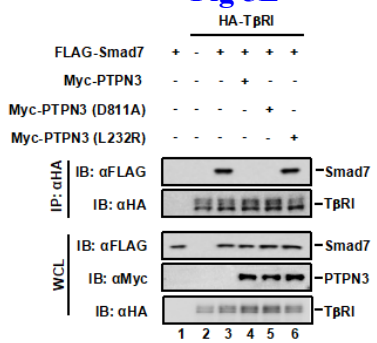
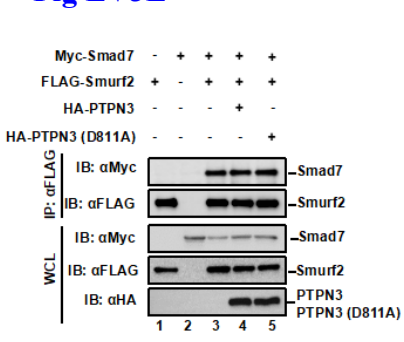


Fig EV3E



3. Since PTPN3(L232R) does not compete with Smad7 to bind to TβRI (which differs from wildtype PTPN3), the failure of PTPN3(L232R) to block the Smurf2-TβRI interaction is then easy to understand. It is conceivable that the L232R mutant adopts a conformation that significantly differs from the wildtype PTPN3, so that the L232R mutant bound to TβRI does not block Smad7 binding. Indeed, in our further experiments, we found that PTPN3

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wildtype bound to the GS region of TβRI as deletion of the entire GS region (including upstream α1, GS loop and downstream α2) of TβRI disabled its interaction with PTPN3 (Fig EV3C). Further fine mapping found that PTPN3 wildtype binds to GS loop and α2 region (Fig EV3C). On the contrary, the L232R mutant could still binds TβRI with the entire or part of the GS region deleted (Fig EV4D).

Fig EV3C
(PTPN3 wildtype)

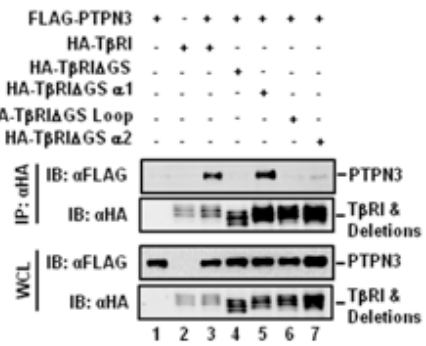
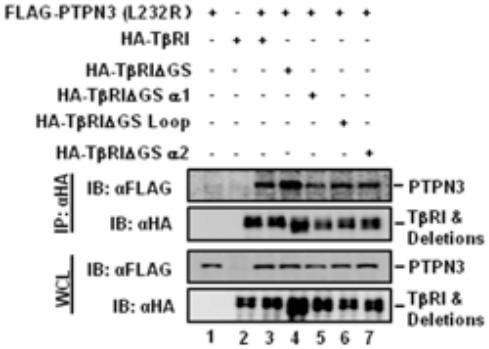


Fig EV4D
(PTPN3 L232R)



Taken together, all these results suggest that the L232R mutant retains the ability to bind to TβRI and meanwhile still recruits the Smurf2-Smad7 complex to TβRI. These are now added to the revised manuscript.

2) Supplementary Figure 2: The authors confirmed that PTPN3(L232R) mutant retained phosphatase activity. This is an important finding and can be mentioned in page 9, where PTPN3 is first described in the main text.

Response: We have revised the manuscript accordingly. While we are the first to report that PTPN3(L232R) still possesses the PPase activity, a previous report by Zheng Y et al. (J Biol Chem 2002;277:42463-42470.) showed that deletion of the FERM domain had no impact on the catalytic activity of PTPN3. The L232 is located in the FERM domain. The original Supplementary Figure 2 is now moved to as Fig 2B.

3) The authors used HeG2 cells in mouse xenograft assays. However, ATCC data sheet says that HepG2 is not tumorigenic in immunocompromised mice. Are the cells really HepG2 cells?

Response: We think the ATCC description is not updated. We also found 778 publications in the PubMed database with search terms "HepG2 and tumors and nude mice". The earliest paper was done by Huber BE et al, who described the tumorigenicity of HepG2 cells in athymic nude mice (Cancer Res. 1985 Sep;45(9):4322-9).

HepG2 cell lines (purchased at different times) are tumorigenic in athymic nude mice, although we have noticed that the tumor growth derived HepG2 cell injection is extremely slow. Yet, knockdown of PTPN3 accelerated tumor development (Fig 7D-F).

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4) Show TbRI blot in Figure 5e. Readers would like to know why Smad2/3 phosphorylation was totally inhibited by PTPN3(L232R).

Response: Yes, high-level expression of PTPN3(L232R) could cause complete inhibition of Smad2/3 phosphorylation (Fig 6A). This is due to the complete degradation of TβRI in the presence of PTPN3(L232R) (Fig 5C).

Fig 6A

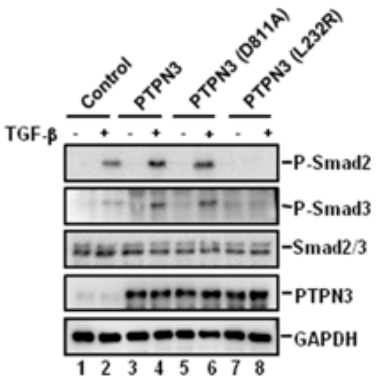
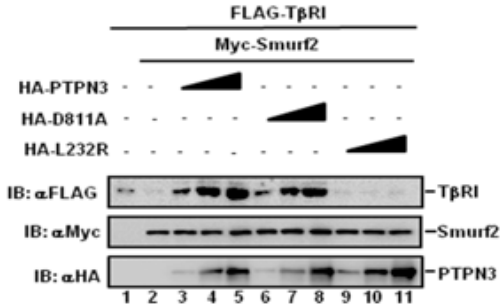


Fig 5C



5) Page 17, Materials and Methods: Delete the section of Immunofluorescence.

Response: This section is retained in the revised paper as new immunofluorescence data are included, per Reviewer #3's suggestion.

6) Figure 1c-f: The authors may have mixed up data for p21 and PAI-1. siRNA-1 completely inhibited TGF-b-induced up-regulation of p21 at 8 h after stimulation in Figure 1c while only partially in Figure 1e. Similarly, siRNA-1 only partially inhibited TGF-b-induced up-regulation of PAI-1 at 8 h after stimulation in Figure 1d while completely in Figure 1f.

Response: Thanks for pointing this out. Indeed, we mixed up the data for p21 and PAI-1. To ensure this, we repeated the experiments again using fresh cells and RT-PCR primers. Below are the new results.

Fig EV1D **Fig 1C** **Fig 1D**

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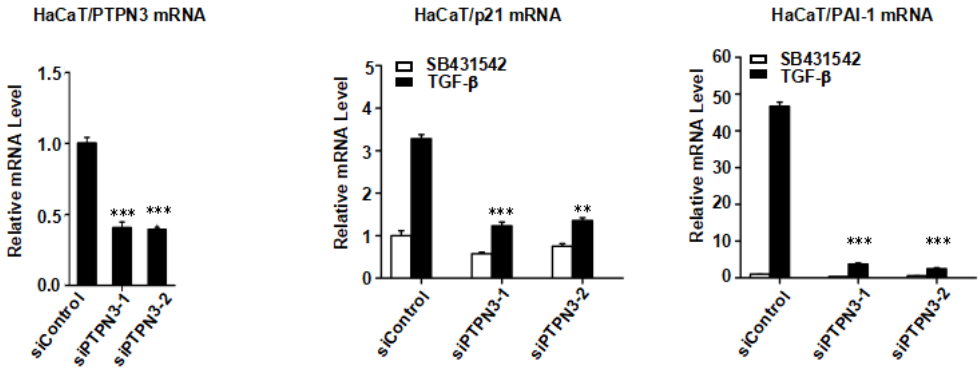
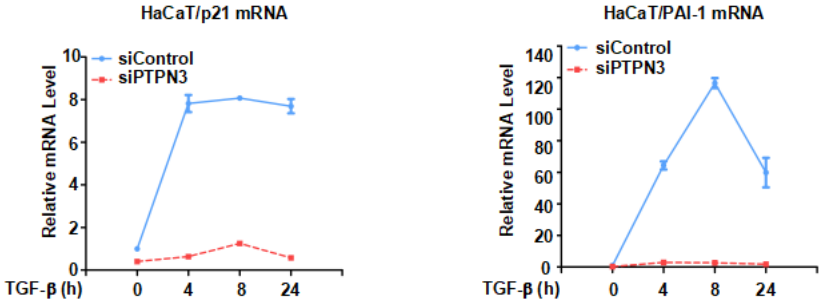


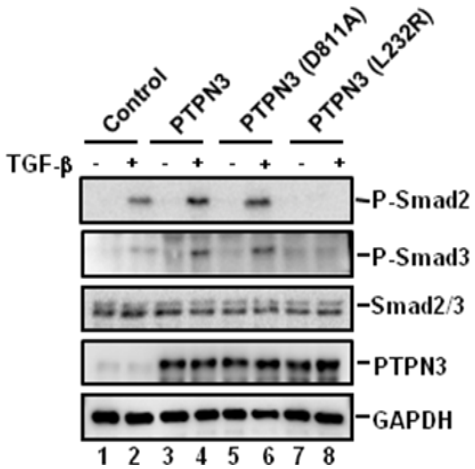
Fig 1E **Fig 1F**



7) Figure 5e: Explain (-) and (+) in the legend.

Response: + or – refers to TGF-β stimulation. This has now been corrected in the new Fig 6A (the original 5E).

Fig 6A



8) Figure 6b and others: Quantification of the results in clonogenic assays (as described in Materials and Methods, page 18) should be presented.

Response: This has properly been revised per the reviewer's instruction. Please see new Fig 6D, F and G.

Fig 6D

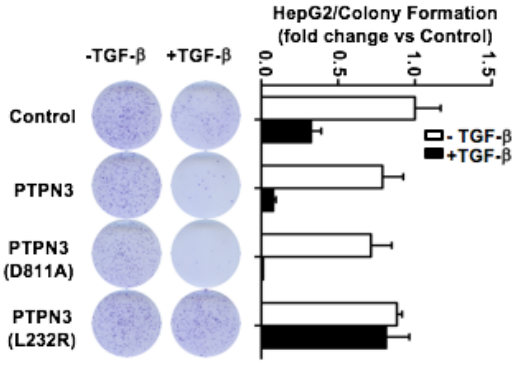
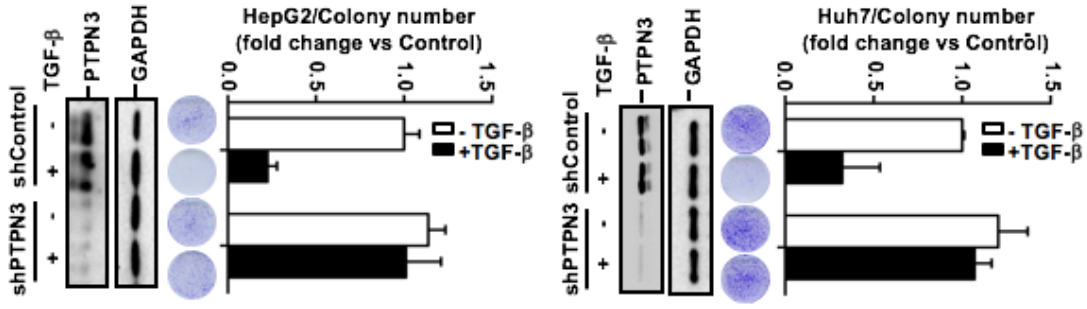


Fig 6F

Fig 6G



9) Figure 6f: Were cells treated with TGF-b? This is not clear.

Response: This has now been revised more clearly. The original Fig 6F is now new Fig 7G. The Western blots were done using tissue lysates from tumors, not cell lines. They were not TGF-β treated. #1 or #2 refers to the tumor # derived from HepG2-induced tumors harboring either shControl or shPTPN3. The data suggest that knockdown of PTPN3 expression attenuated TGF-β signaling in the tumors.

Fig 7G

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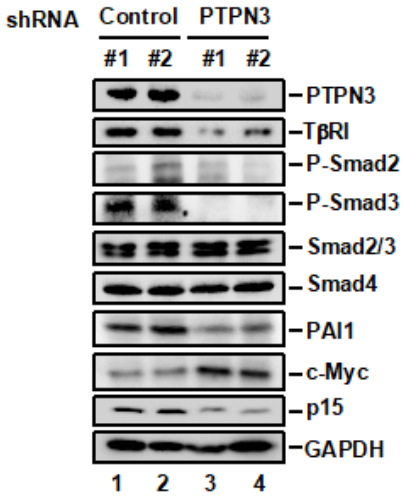

10) Page 4, line 8 from the bottom: liver progression >> liver cancer progression?

Response: This has properly been revised per the reviewer's instruction.

Referee #3:

Yuan and co-authors present a set of experimental approaches that link the action of the PTPN3 tyrosine phosphatase to regulation of signaling by the receptor kinase for the cytokine transforming growth factor beta (TGFbeta). Overall, the main conclusions of this paper are interesting with respect to their importance in the regulation of TGFbeta signal transduction. The approaches are well established in the field and in the laboratory of Dr. X-H. Feng and involve primarily biochemical interaction assays in transfected cell models. Similar to PTPN3, the Feng and other labs have previously established numerous independent regulators of TGF-beta signaling. The novelty of the report on PTPN3 is that this protein is an established tyrosine phosphatase, which acts as a TGF-beta receptor regulator in the absence of its catalytic activity. This interesting finding and the identification of a genetic mutation in PTPN3 (L232R) that does not affect phosphatase activity, yet it disrupts the regulation of the TGF-beta receptor provide exciting ground based on which the authors may build a deeper analysis of the mechanism by which PTPN3 regulates the TGF-beta receptor. In view of the current understanding of regulation of TGF-beta signaling, this appears to be a necessary additional set of experiments that will explain deeper the function of PTPN3 as a TGF-beta regulator.

1. Essentially all biochemical experiments use transfected proteins. Is it possible to demonstrate the endogenous complex between PTPTN3 and TbetaRI?

Response: We have carried out the co-IP between PTPN3 and TβRI without transfections. As shown Fig 4A, we found that PTPN3 and TβRI could interact with each other with both at the endogenous levels in HaCaT cells.

Fig 4A

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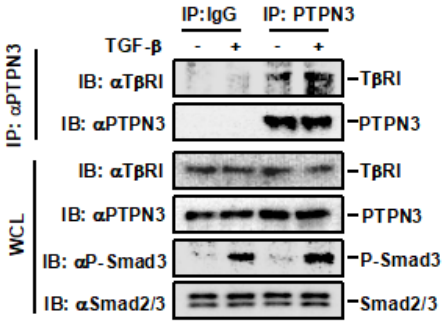


2. Does shRNA against the endogenous PTPN3 interfere with the interaction between Smurf2 and the TbetaRI?
3. Smurf2 is thought to bind the TGFbeta receptor via the adaptor protein Smad7. Does Smad7 make a complex with PTPN3 or is the interaction between PTPN3 and the receptor direct?
4. Which part of the TBetaRI is involved in the interaction with PTPN3?

Response: To answer these questions, we performed additional experiments. We already showed that overexpression of PTPN3 could attenuate the association between Smurf2 and TβRI (Fig 4F, 5D). Because Smurf2 is brought to the TβRI by Smad7 (Mol Cell. 2000; 6: 1365-75.), overexpression of PTPN3 could indeed block the Smad7-TβRI interaction (Fig 5E). Furthermore, we examined the effect of PTPN3 shRNA on the interaction between Smad7 and TβRI. We found that shRNA-mediated knockdown of the PTPN3 expression enhanced the Smad7-TβRI interaction (Fig 4E).

Fig 5E

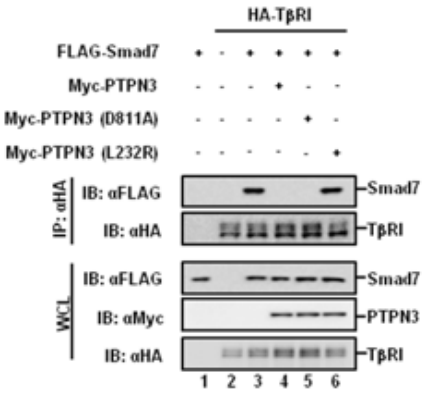
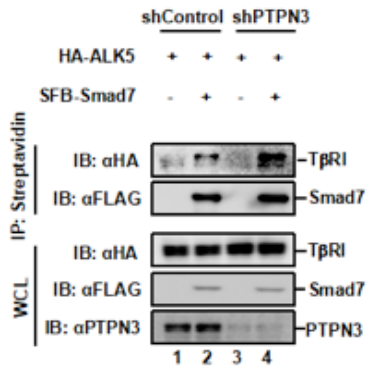


Fig 4E



We have mapped the PTPN3-binding domain to the GS loop-α2 region of TβRI (aa 185-205) (Fig 4D, EV3A and EV3C), which overlaps with Smad7-binding region (Fig EV3D). Consistently, PTPN3 abolished the binding of Smad7 to TβRI (Fig 5E), explaining how PTPN3 enhances TβRI function. Interestingly, the L232R mutant still binds to TβRI (Fig 5D), yet (surprisingly) it does not block the binding of Smad7 or Smurf2 to TβRI (Fig 5D and E: 5E is new data), suggesting that L232R binds to TβRI in a different mode.

Fig 4D **Fig EV3A**

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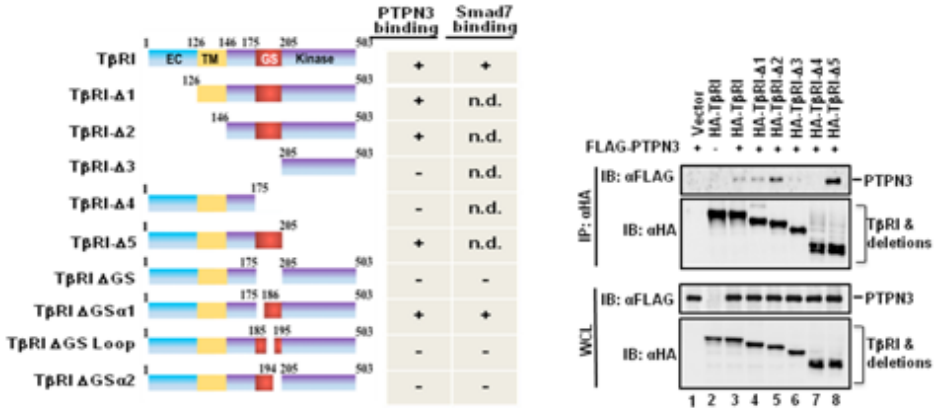
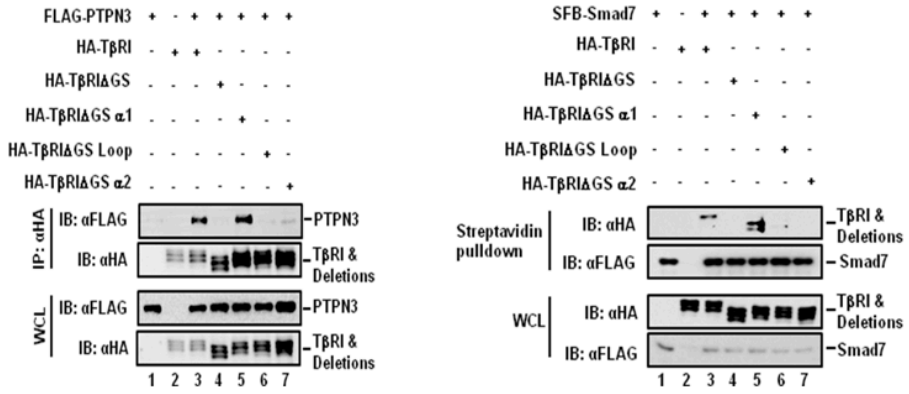


Fig EV3C **Fig EV3D**



5. How does the FERM domain and specifically Leu232 regulate the TGF-beta receptor? This requires further analysis? Does this interaction take place on the plasma membrane or on endosomal membranes?

Response: The FERM domains are often associated with proteins located at the plasma membrane or at the interface between the plasma membrane and the cytoskeleton. Per the reviewer's suggestion, we performed immunofluorescence

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experiments. We found that PTPN3 wildtype, phosphatase-dead D811A and the mutant L232R shared similar patterns of subcellular localization at the plasma membrane and some in the cytoplasm (Fig 5A). In addition, the isolated FERM domain or the FERM domain with L232R mutation had similar subcellular localization (Fig EV4A).

Fig 5A

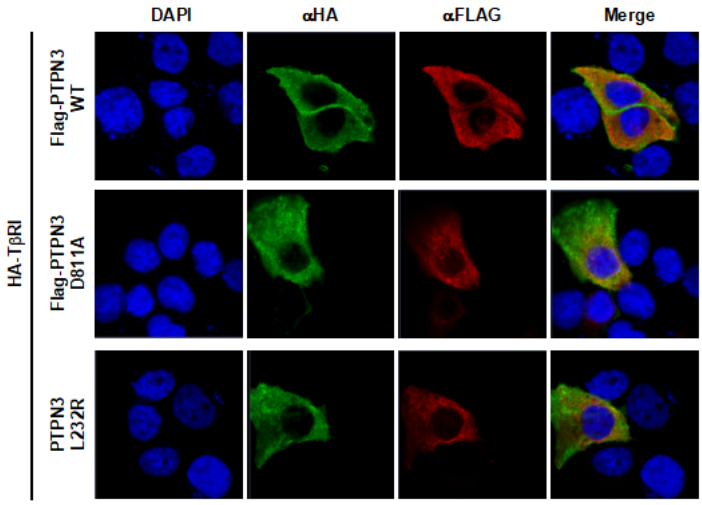
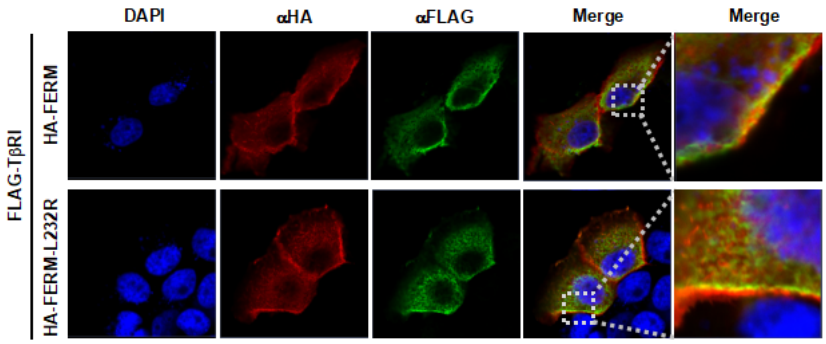


Fig EV4A



6. Since L232R occurs in ICC, is it possible to analyse TGF-beta signaling in samples from such patients by immunohistochemistry or RNA-based methods? Is TGF-beta cytostatic or pro-tumorigenic in cholangiocytes?

Response: We performed in silico gene profiling analysis based on public databases to delineate the relationship of PTPN3 with TGF-β signaling in ICC tumor tissues. However, we did not get clear relationship between PTPN3 and TGF-β signature. It is possible that we cannot verify these samples clearly about which variant of PTPN3 they have, so the analysis might mix the relationship between PTPN3 WT and TGF-β signature with the relationship between PTPN3 (L232R) and TGF-β signature. The role of TGF-β signaling in different stages of ICC is not easy to distinguish. Somatic mutant or depletion of Smad4 is common in

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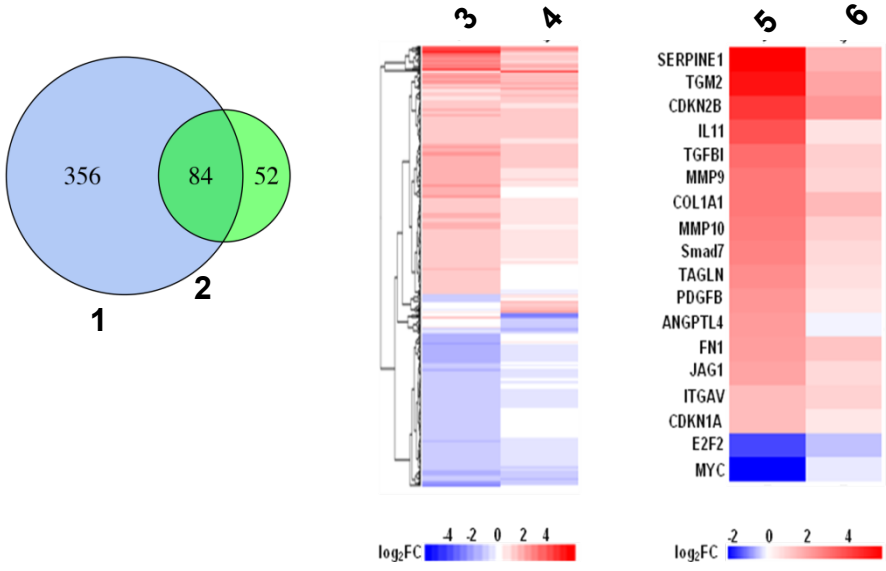
cholangiocarcinoma and loss of TGF-β receptor II has also been found in ICC (Nat Genet 2012; 44: 690-3. Nat Genet 2013; 45: 1470-3). TGF-β signaling inhibits cholangiocyte proliferation at later stages so that it attenuates the development of cholangiocarcinoma arising from hepatocytes and cholangiocytes (Gastroenterology 2015; 150 (3), 720–733). Our observations that the PTPN3(L232R) dampens TGF-β signaling support the notion that PTPN3 fits well as a tumor suppressor as other components in the TGF-β pathway.

7. Since PTPN3 regulates TGF-beta receptor signaling, all responses to TGF-beta are affected by PTPN3. This is necessary to demonstrate beyond the 3 genes examined (p21, myc, PAI-1) and cell proliferation. Does PTPTN3 affect all gene responses to TGF-beta?

Response: To examine if PTPN3 affects all gene responses to TGF-β, we carried out an RNA-Seq experiment in HaCaT with siPTPN3 and siControl. RNA-seq analyses showed that 440 genes were up- or down-regulated (Fold change > 2) upon TGF-β treatment in parental HaCaT cells, whereas only 84 of them were responsive to TGF-β in the siPTPN3 cells (Fig 1I-K), indicating that PTPN3-depleted cells profoundly lost responsiveness to TGF-β in gene transcription. Next, we specifically examined a group of known TGF-β target genes, including *SERPINE1, TGM2, CDKN2B, IL11, TGFBI, MMP9, COL1A1, MMP10, SMAD7, TAGLN, PDGFB, ANGPTL4, FN1, JAG1, ITGAV, CDKN1A, E2F* and *MYC*. As shown in Fig 1K, TGF-β induced upregulation or repression of most of the selected target genes in control cells, but not or to a lesser extent in the PTPN3-depleted cells. Together, our genome-wide transcriptional analyses supported the positive role of PTPN3 in TGF-β responses. These data are presented in Fig 1I-K in the revised paper.

Fig 1I **Fig 1J** **Fig 1K**



8. Is the action of PTPN3 specific for epithelial cells or does it also regulate TGF-beta signaling in fibroblasts, lymphocytes or stem cells?

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Response To address this issue, we have examined the effect of PTPN3 knockdown on expression of TGF-β target genes. As shown below, near 75% depletion of PTPN3 in mouse fibroblast L929 cells attenuated TGF-β-mediated upregulation of fibronectin and downregulation of E-cadherin (Fig EV1H). Similarly, PTPN3 knockdown abolished TGF-β-induced expression of fibronectin and N-cadherin in MRC-5 cells. Thus, PTPN3 also functions to promote TGF-β signaling in fibroblasts (Fig EV1I).

Fig EV1H

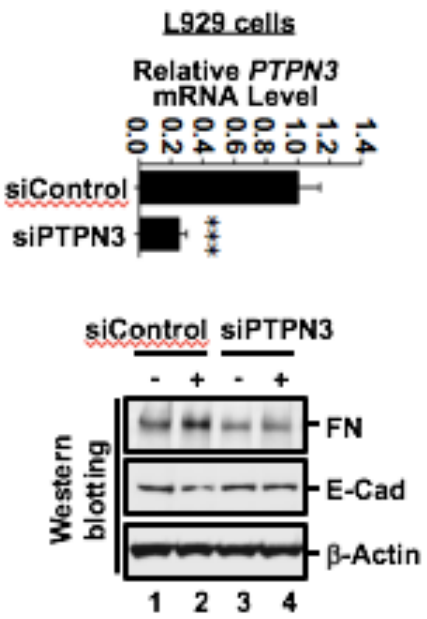
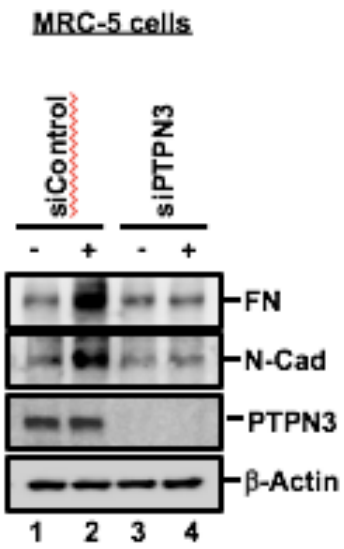


Fig EV1I



9. The methods will be more complete if the analysis of TCGA expression data and Kaplan-Meier analysis is explained.

Response: Kaplan-Meier method was used to calculate survival curves and used a log-rank test to check whether gene levels were significantly associated with overall patient survival. Patient samples were grouped into high and low expression

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group based on medium expression of PTPN3. Student *t*-test was used to compare PTPN3 mRNA levels between normal and tumor samples from The Cancer Genome Altas (TCGA) liver cancer database.

10. The acknowledgments describe a series of human liver cancer cell lines, which are not described in the methods or results. The acknowledgements can be amended accordingly.

Response We appreciate the reviewer's suggestion. We have amended the acknowledgements accordingly.

11. The discussion has large sections that simply repeat the results and reiterate many times the same points. The discussion on TGF-beta being tumor suppressive or pro-tumorigenic and PTPN3 having similarly dual roles is confusing. I thought that the paper tries to make a case that PTPN2 acts in a tumor suppressive manner. This part of the discussion can be clarified better or omitted.

Response: We greatly appreciate the reviewer's suggestion. We hope we now have much improved discussion.

2nd Editorial Decision 7th Mar 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by all of the original referees whose comments are shown below.

As you will see, the referee finds that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before I can send the official acceptance letter, there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS:

Referee #1:

In the revised manuscript, the authors have provided a number of detailed experiments and interpretations in response to my comments. Specifically, the authors have acquired similar results from other HCC cell lines. Moreover, the additional in vivo experiment showed that PTPN3-L232R mutant exhibited tumor promotive effect on HCC tumor growth. Overall, the authors have satisfactorily addressed my concerns.

Referee #2:

In this revised manuscript, the authors have deepen mechanistic studies and well addressed concerns raised by reviewers. I have no additional comments.

Referee #3:

In the revised paper Yuan and colleagues provide a significantly superior account of work that demonstrates the novel function of the phosphatase PTPN3, as a regulator of TGF-beta receptor signaling in a variety of cell types. PTPN3 positively contributes to TGF-beta and a detailed mechanism of its action, which bypasses the requirement of catalytic phosphatase activity, is established in this paper. Furthermore, biological significance of the new regulatory mechanism points to intrahepatic cholangiocarcinoma, where, mutant forms of PTPN3 can be explained based on suboptimal TGF-beta signaling.

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By responding to all comments by this reviewer, but also sufficiently well to comments by other reviewers, and based on the novelty of the original observation reported in this paper, and the impressive effort made to enhance the molecular and biological mechanism reported in depth, it is evident that this paper will generate a serious and widespread interest in the fields of signal transduction and cancer biology, and will be deeply appreciated by the very wide network of TGF-beta signaling experts.

2nd Revision - authors' response 14th Mar 2019

The authors performed all requested editorial changes.

3rd Editorial Decision 28th Mar 2019

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in The EMBO Journal.

Congratulations on the very nice work!

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: Xin-Hua Feng
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99945R

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?	We performed all experiments with at least three independent samples. We used more than the minimum number of mice for analysis in the study according to 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 did not use any statistical method to predetermine sample size in the animal studies
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were neither sample nor 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.	No.We assigned the animals randomly. All the animals or samples processed in the same manner
For animal studies, include a statement about randomization even if no randomization was used.	We did not use any statistical method to predetermine sample size in the animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were performed in a blinded manner
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.	Yes. We used a two-tailed ,unpaired t-test for the statistical analysis and P<0.05 was considered statistically significant
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

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).	Vendor name and catalog number of all the antibodies we used are provided in Materials 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.	293T was from ATCC. HaCaT was from Norbert Fusening. Source of the other cell lines were described in the text. All the cells were in good conditions but we did not test 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.	Nude mice were purchased from Shanghai SLAC Laboratory Animal Company.5-week-old nude mice including both male or female were used in the study. Mice were housed in the enviroment of 20-22℃, with a 12/12 hours light and dark cycle
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 confirm

E- Human Subjects

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

F- Data Accessibility

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	We have deposited the original RNA-seq datasets: Gene Expression Omnibus GSE127903.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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