

Manuscript EMBO-2016-43270

## USP26 regulates TGF- $\beta$ signaling by deubiquitinating and stabilizing SMAD7

Sarah Kit Leng Lui, Prasanna Vasudevan Iyengar, Patrick Jaynes, Zul Fazreen Bin Adam Isa, Brendan Pang, Tuan Zea Tan, and Pieter Johan Adam Eichhorn

*Corresponding author: Pieter Johan Adam Eichhorn, Cancer Science Institute of Singapore*

---

<b>Review timeline:</b>	Submission date:	26 August 2016
	Editorial Decision:	19 October 2016
	Revision received:	12 January 2017
	Editorial Decision:	22 February 2017
	Revision received:	01 March 2017
	Editorial Decision:	07 March 2017
	Accepted:	08 March 2017

---

Editor: Martina Rembold /Achim Breiling

### 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

19 October 2016

Thank you for the submission of your research manuscript to EMBO reports and for your patience while the manuscript was under review. We have now received the full set of referee reports that is copied below.

While all three referees acknowledge that the findings are potentially interesting, they all point out that significant revisions are required and that the functional link between USP26 and the TGF $\beta$  receptor and TGF/Smad signaling need to be strengthened before the study can be considered for publication here. The referees raise concerns about the fact that overexpressed proteins are used throughout the study and upon further discussion with the referees all agree that protein stability should be measured in stable cell lines, although these need not to be tumor cell lines. The referees are also concerned that no evidence is provided that USP26 controls endogenous Smad7 and TbRI.

Upon further discussion with referee 2 s/he also specified the more general comments listed as major points 3 and 4 in more detail and suggests the following experiments:

**Point#3:** The authors should genetically disrupt the expression of USP26 by shRNA in the glioblastoma cells and then check the ubiquitination status as well as protein stability of SMAD7. The utilization of USP26 inhibitors to address this point is encouraged but not obligated.

**Point#4:** The authors should check the activity of downstream effectors in the TGF $\beta$  pathways in control cells and USP26-depleted cells in response to TGF $\beta$  stimulation. It would help to understand the role of USP26 in TGF $\beta$  pathway in general.

From these comments it is clear that publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further and extend this deadline, 4 months might be more realistic.

-----

## REFEREE REPORTS

### Referee #1:

In this manuscript, the authors reveal that USP26, a deubiquitinating enzyme, participates in a negative feedback loop to modulate TGF- signaling by deubiquitinating and stabilizing SMAD7. Mechanistically, USP26 acts as a SMAD7 ubiquitin chain specific deubiquitylase to protect SMAD7 from proteasome-mediated degradation pathway. This then facilitates SMAD7-SMURF2 conjunction promoting TGF- receptor complex degradation and leading to reduced TGF- activity. Moreover, knockdown of USP26 enhanced TGF- induced migration in tumor cells. Overall, the authors identified USP26 as part of a negative feedback loop regulator of the TGF- pathway. There are some concerns that need to be addressed.

#### Major concerns:

1. The authors claim that USP26 is selected by a shRNA deubiquitinating enzyme screen, and they finally confirm that knockdown of USP26 greatly enhanced TGF- activity (Figure 1A and 1C). To draw this conclusion more compelling, one or two excluded DUB(s) should be employed as a negative control in Figure 1A.
2. In Figure 1B, the authors show inhibitory effect of shRNA vectors on USP26 by using GFP antibody to measure the exogenous GFP-USP26 fusion protein expression, for the reason that they failed to find appropriate antibody detecting endogenous USP26 in Western blotting assay. To verify endogenous USP26 is inhibited by the corresponding shRNAs, mRNA of endogenous USP26 should be measured without GFP-USP26 overexpression conditions.
3. It shows that USP26 knockdown apparently enhanced TGF- induced migration and invasion (Sup. Figure 8A-D). On the other hand, the authors should confirm whether overexpression of USP26 has an inhibitory effect on TGF- induced migration.
4. IHC staining of tumor samples with high p-SMAD2 level and low USP26 expression should be presented compared to that of existing tumor sections in Figure 4B.
5. To support the working model shown in Figure 4D, the authors should provide further evidence to show that SMURF2 accelerates SMAD7 ubiquitination and degradation in USP26 knockdown cells.

#### Minor comments:

1. Endogenous SMAD7 should be presented by anti-SMAD7 antibody in Figure 3C, 3D and Sup. Figure 5A, 5B.
2. In Figure 3C and 3D, the authors should explain why the "Flag signaling" is positive in the first column where the control vector, but not flag-SMAD7, is transfected into 293T cells.

3. Proteasome inhibitor MG132 is used to block ubiquitination-mediated protein degradation, which helps to determine the different levels of ubiquitinated protein between each group in IP assays. Therefore, this critical condition should be indicated in Figure 3A, 3H and Sup. Figure 2A, 2B.

#### **Referee #2:**

In this manuscript, Lui et al. reported USP26 as a key component of the TGF $\beta$  negative feedback loop by deubiquitinating the TGF $\beta$  RI inhibitor SMAD7. They found that knock-down of USP26 upregulated the TGF $\beta$  signaling, which was mediated by increased phosphorylation of SMAD2. Consistently, overexpression of USP26 inhibited TGF $\beta$  signaling and suppressed SMAD2 phosphorylation. By a systematic analysis of the affinity of USP26 to 7 SMADs, they found that USP26 interacted with SMAD7. They revealed that knock-down of USP26 increased the ubiquitination of SMAD7, leading to decreased SMAD7 proteins. However, knock-down of USP26 decreased the ubiquitination of TGF $\beta$  RI, which is regulated by SMAD7. Therefore, they concluded that USP26 deubiquitinates SMAD7, which in turn upregulates ubiquitination and degradation of TGF $\beta$  RI. Finally, they showed that in glioblastoma tissues, USP26 signal inversely correlated with pSMAD2 signal. In addition, high USP26 expression favors GBM patient prognosis, suggesting some clinical significance of USP26.

The regulation of the TGF $\beta$  pathway through the DUB mediated stability of the key component in the TGF $\beta$  negative feedback loop is an interesting point. Whereas the logic is attractive, more data are required to make the conclusion solid. Furthermore, the importance of the USP26 on the whole TGF $\beta$  pathway signaling should be clarified.

#### Major concerns:

1. The authors tended to use overexpression in 293FT cells throughout the manuscript to address the biochemical mechanisms. Although it is okay to determine the interactions between proteins, it is vague to determine the stability of the proteins affected by ubiquitination. The overexpressing efficiency itself may affect the protein levels. Since the authors mentioned in the manuscript that they had established some stable cell lines, they may want to address the protein stability issue by using these stable cell lines.
2. The authors used a lot of qPCR assays to determine the effect of USP26. However, USP26 is a DUB that functions on the post-translational level. The authors may want to show the protein levels to clarify the function of USP26.
3. The ubiquitination assays lacked some critical experiments.
4. Whereas the effect of USP26 on SMAD7 was clearly demonstrated, the effect of USP26 on the TGF $\beta$  pathway in general was not that clear.

#### Other concerns:

1. The authors showed that USP26 mRNA was upregulated after TGF $\beta$  treatment. They may want to determine the protein levels of USP26 before and after TGF $\beta$  treatment in tumor cells.
2. The upregulation of SMAD7 proteins by knock-down of USP26 was only determined in 293FT overexpressing system. It should be determined in tumor cells by detection of the endogenous SMAD7 proteins.
3. The authors used a reporter system to show the upregulation of TGF $\beta$  signaling by knock-down of USP26. They should provide some evidence of the activation of TGF $\beta$  pathway in tumor cells by checking the downstream targets of TGF $\beta$  pathway.
4. Since the authors used overexpression of USP26 in 293FT cells in a lot of experiments, they may want to present the endogenous and ectopic USP26 protein levels side by side.
5. Fig 2D, given that the input of USP26 is much less in the pull-down assay of SMAD3, it is possible that SMAD3 had the similar affinity to USP26. The authors may want to clarify it.
6. Fig 3 C, D, F, and G used overexpression of multiple plasmid to address the stability of a certain protein, which is questionable. It would be better to determine the stability of the endogenous target proteins either in the stable cell lines or in tumor cell lines.
7. Fig 3E the actin input was not even, which made the trend of SMAD7 proteins questionable. It is also strange that the TGF $\beta$  stimulation was applied in this assay. Fig 3A and B showed a strong UB signal without TGF $\beta$ , suggesting that TGF $\beta$  stimulation is not required to trigger the degradation of SMAD7.
8. Whereas USP26 knock-down upregulated the ubiquitination of SMAD7, overexpression of USP26 as a DUB to downregulate the ubiquitination of SMAD7 should be presented.

9. Fig 1D showed that KD of USP26 dramatically upregulated SMAD7 mRNA levels. Is it possible that in tumor cells, lack of USP26 may trigger the upregulation of SMAD7 transcription to compensate for the loss of SMAD7 proteins? The authors may want to do the immunohistochemical analysis of the tumor tissues used in Fig 4 to address the question.

10. Could the authors perform a combined analysis of TGF- $\beta$  and USP26 by using the REMBRANDT database to clarify the importance of USP26 in TGF- $\beta$  pathway? They could compare the patient prognosis of TGF High/LowUSP26High/Low groups (4 groups).

### Referee #3:

The authors report that the de-ubiquitylase USP26 controls the stability of the inhibitory Smad7, which is known to bring Smurf2 (and perhaps some other E3 ubiquitin ligases) to the type I TGF- $\beta$  receptor (TbRI) and thus promotes TbRI degradation. Consequently, USP26 is seen as a determinant of TGF- $\beta$  signaling. The authors also correlate loss of USP26 with high TGF- $\beta$  activity and poor prognosis in glioblastomas.

Overall, the authors show some interesting results that highlight the potential role of USP26 as a de-ubiquitylase for Smad7 that controls TGF- $\beta$  signaling. This report adds to previous reports on other de-ubiquitylases that control TGF- $\beta$  signaling. The data shown are convincing. Where I have a problem is that overall this story is not very well worked out. Specifically, I am missing convincing evidence that (1) USP26 controls Smad7 and TbRI stability at endogenous levels of these signaling mediators, (2) that USP26 is functionally linked to ubiquitylation of TbRI by Smurf2. These aspects should be better worked, realizing very well that these experiments are harder than those shown.

#### Comments in order of appearance in the manuscript:

- The authors use phosphoSmad2 as read-out of TGF- $\beta$ -induced Smad activation, and then look at transcription targets that are activated by Smad3. Since Smad3 is the major Smad effector for TGF- $\beta$ , some data should show the effect of USP26 on Smad3 activation.
- Can the authors show that USP26 associates with endogenous Smad6 or Smad7 (rather than cotransfected, overexpressed proteins)? Is this endogenous interaction ligand-induced?
- The authors focus on Smurf2 as E3 ubiquitin ligase that associates with Smad7. I thought that under the same experimental conditions also the Smurf1 E3 ligase would associate. So, related to Fig. 2F, does USP26 show up in the complex with Smad7 and Smurf1?
- Related to the functional link of USP26 with Smurf2-mediated ubiquitylation, what is the effect of increased or decreased USP26 expression on Smad7 levels when Smurf2 expression is downregulated? And what happens to the TbRI levels under these circumstances?
- Can the authors show that USP26 controls endogenous Smad7 levels?
- Does increased or decreased USP26 expression affect the levels of Smad7, when proteasomal degradation is inhibited. This should not be the case, if the authors are right, but such data need to be shown in conjunction with Fig. 3C, D.
- Does increased or decreased USP26 expression affect the endogenous TbRI levels, and what is the effect on cell surface TbRI? This is of key importance, but not addressed.

#### Minor:

- page 3, sentence starting at line 6 from bottom: The authors see Smad7 primarily as a scaffold to recruit E3 ubiquitin ligases, such as Smurf2, to promote TbRI degradation. While this statement may serve the authors well to present their findings, most researchers in the field would see Smad7 primarily as an inhibitory Smad that prevents R-Smad activation.
- Some incorrect or awkward sentences can be found. Maybe read over it one more time to further polish the text.
- Panels 2A and 2B are results from close to identical experiments with the same results. Only one of these should be shown.
- page 7, first sentence of first full paragraph: This statement is an exaggeration.
- On page 10, halfway: Mark that the Discussion starts there.

Responses to reviewer's comments:

We thank the reviewers for the thoughtful and thorough revision of the manuscript. Thanks to their insights and comments our manuscript has greatly improved. We are glad to report that we have addressed the majority of the concerns and comments raised by the reviewers. We have now 27 new figure panels (Figs. 1C, 2E, 2F, 3C, 3D, 3E, 3H, 3I, 4E, 4F, 4G, 5B, 5C, EV1A, EV1B, EV1C, EV1G, EV2A, EV2B, EV2C, EV2D, EV2G, EV3E, EV5B, EV5C, EV5D, EV5E).

**Reviewer #1:** *The authors claim that USP26 is selected by an shRNA deubiquitinating enzyme screen, and finally confirm that knockdown of USP26 greatly enhanced TGF- $\beta$  activity (Figure 1A and 1C). To draw this conclusion more compelling, one or two excluded DUB(s) should be employed as a negative control in Figure 1A.*

We thank the reviewer for their critical reading of the manuscript. We have provided the original screen and corresponding table demonstrating the individual DUB activity on the CAGA-Luc reporter. This experiment demonstrates the enhanced luciferase activity of DUB51 pool (USP26). **(New figure EV1A, B)**

**Reviewer #1:** *In Figure 1B, the authors show inhibitory effect of shRNA vectors on USP26 by using GFP antibody to measure the exogenous GFP-USP26 fusion protein expression, for the reason that they failed to find appropriate antibody detecting endogenous USP26 in Western blotting assay. To verify endogenous USP26 is inhibited by the corresponding shRNAs, mRNA of endogenous USP26 should be measured without GFP-USP26 overexpression conditions.*

We thank the reviewer for bringing up this point. We now demonstrate knockdown of endogenous USP26 by hairpins shUSP26-1 shUSP26-2. **(New figure 1C)**

**Reviewer #1:** *It shows that USP26 knockdown apparently enhanced TGF- $\beta$  induced migration and invasion (Sup. Figure 8A-D). On the other hand, the authors should confirm whether overexpression of USP26 has an inhibitory effect on TGF- $\beta$  induced migration.*

Following the reviewers suggestion we generated MDA-MB-231 cells ectopically expressing GFP-USP26 or its corresponding GFP-USP26 DD mutant. We show that overexpression of USP26 decreased the invasion capacity of MDA-MB-231 cells following exposure to TGF- $\beta$ . An effect which was diluted in USP26 mutant cell line. **(New figure 4E, F, G).**

**Reviewer #1:** *IHC staining of tumor samples with high p-SMAD2 level and low USP26 expression should be presented compared to that of existing tumor sections in Figure 4B.*

We agree with the referee and we thank him for raising this point. We have now included a patient sample, which more adequately demonstrates low USP26 and corresponding high pSMAD2. **(New Figure. 5B)**

**Reviewer #1:** *To support the working model shown in Figure 4D, the authors should provide further evidence to show that SMURF2 accelerates SMAD7 ubiquitination and degradation in USP26 knockdown cells.*

We would like to thank the reviewer for bringing up this issue as it gives us the opportunity to discuss this point in detail and apologise for any lack of clarity within our discussion. We had originally stated in the discussion, "As SMURF2 does not directly target SMAD7 for ubiquitination [...] and [...] that USP26 counteracts the ubiquitination and degradation of SMAD7 by another E3 ligase in the cytoplasm while in complex with SMURF2". Previously it has been reported by others that SMURF2 does not directly appear to target SMAD7 for ubiquitination and degradation [1], but rather in this context SMAD7 recruits SMURF2 to the TBR complex whereby SMURF2 targets the entire TBR-SMAD7-SMURF2 complex for degradation partially through proteosomal mediated mechanisms (lipid raft) but also dependent through lysosomal dependent mechanisms. Importantly for this story, it has recently been demonstrated that NEDD4 ubiquitin ligase family members,

including SMURF2, act predominantly as lysine 63 ligases [2-4]. We demonstrate that USP26 is a lysine 48 specific deubiquitinase. **We therefore believe that the role of USP26 is to counteract the ubiquitination and degradation of SMAD7 by another E3 Lys48 specific ligase in the cytoplasm (not SMURF2) while in complex with SMURF2.** To further demonstrate this point we analyzed if SMURF2 overexpression enhanced SMAD7 Lys48 incorporation in the presence or absence of knockdown vectors targeting USP26. As can be seen below SMURF2 decreased Lys48 ubiquitination of SMAD7 and once again depletion of USP26 significantly enhanced Lys48 ubiquitination of SMAD7. The fact that SMURF2 decreases Lys48 ubiquitination is quite a surprising result and will need to be explored in greater detail.

- [1] Ogunjimi et al. Molecular Cell 19, 297-308, August 5, 2005  
 [2] Maspero et al. Nature Structural Molecular Biology 6, 696-70, June, 2013  
 [3] Berndsen and Wolberger. Nature Structural Molecular Biology 21, 301-307, April, 2014  
 [4] Kim et al. Mol. Cell Biology 29, 3307-3318, 2009.

We would like to address the following question from Reviewer number 3 along with the question above.

**Reviewer #3:** *Related to the functional link of USP26 with Smurf2-mediated ubiquitylation, what is the effect of increased or decreased USP26 expression on Smad7 levels when Smurf2 expression is downregulated?*

In respect to the suggestions above we do not believe that USP26 counteracts the ubiquitination of SMAD7 by SMURF2 as SMURF2 is Lys63 ligase while USP26 is Lys48 deubiquitinase. Nevertheless, we performed the experiments as suggested by both reviewers. To our surprise knockdown of SMURF2 decreased SMAD7 expression, which was further decreased by knockdown of USP26. As this was completely unexpected we performed this experiment in three independent cell lines stably knocked down for USP26 and observed similar results. We also observed this result in SMURF2 CRISPR knockout cell lines.

[Data not included in peer review process file.]

Therefore, in the answer to the reviewer's questions we do not believe that SMURF2 accelerates SMAD7 ubiquitination or degradation in USP26 knockdown cells. But rather, through the reviewer's suggestions we have now described a completely unexpected novel mechanism for SMAD7 stability whereby SMURF2/SMAD7 complex formation somehow appears to stabilize SMAD7. The details of this mechanism will be explored in future studies. With the reviewer's permission we would like to exclude this data from this manuscript as it does not add to our original argument that USP26 acts as a Lys48 deubiquitinase counteracting the function of a E3 ligase, not SMURF2, resulting in the stability of SMAD7. We have also revised the discussion section to include these points in greater detail.

**Reviewer #1:** *Endogenous SMAD7 should be presented by anti-SMAD7 antibody in Figure 3C, 3D and Sup. Figure 5A, 5B.*

We agree with the reviewer. We now demonstrate that knockdown of USP26 decreases endogenous SMAD7 levels. (**New Figure 3C, 3E**).

**Reviewer #1:** *In Figure 3C and 3D, the authors should explain why the "Flag signaling" is positive in the first column where the control vector, but not flag-SMAD7, is transfected into 293T cells.*

We regret the lack of clarity in our figures. We consistently observe a background band at 46 kDa when using FLAG rabbit antibodies. This has now been denoted in the respective figures. (**Fig. 3A, B. Fig. EV3E,F**).

**Reviewer #1:** *Proteasome inhibitor MG132 is used to block ubiquitination-mediated protein degradation, which helps to determine the different levels of ubiquitinated protein between each group in IP assays. Therefore, this critical condition should be indicated in Figure 3A, 3H and Sup.*

Figure 2A, 2B.

We thank the reviewer for pointing out our omission. We have now included this detail where appropriate.

**Reviewer #2:** *The authors tended to use overexpression in 293FT cells throughout the manuscript to address the biochemical mechanisms. Although it is okay to determine the interactions between proteins, it is vague to determine the stability of the proteins affected by ubiquitination. The overexpressing efficiency itself may affect the protein levels. Since the authors mentioned in the manuscript that they had established some stable cell lines, they may want to address the protein stability issue by using these stable cell lines.*

We thank the reviewer for their time in reading this manuscript. We now demonstrate that loss of USP26 degrades endogenous SMAD7 and stabilizes endogenous TGF- $\beta$  receptor I (**New Figure 3C, 3E, 3H**)

**Reviewer #2:** *The authors used a lot of qPCR assays to determine the effect of USP26. However, USP26 is a DUB that functions on the post-translational level. The authors may want to show the protein levels to clarify the function of USP26.*

We thank the reviewer for their suggestion. Unfortunately, we were unable to identify an antibody that effectively detected endogenous USP26 by western blot.

**Reviewer #2:** *The authors should genetically disrupt the expression of USP26 by shRNA in the glioblastoma cells and then check the ubiquitination status as well as protein stability of SMAD7. The utilization of USP26 inhibitors to address this point is encouraged but not obligated.*

We like to thank the reviewer for this excellent suggestion. For the reviewers information we analyzed endogenous ubiquitination levels of endogenous SMAD7 in the glioblastoma primary cultured human cell line and observed that in cells depleted for USP26 the levels of incorporated ubiquitin on SMAD7 increased. These results are in line with our previous results in 293T cells.

[Data not included in peer review process file.]

**Reviewer #2:** *The authors should check the activity of downstream effectors in the TGF $\beta$  pathways in control cells and USP26-depleted cells in response to TGF $\beta$  stimulation. It would help to understand the role of USP26 in TGF $\beta$  pathway in general.*

We thank the reviewer for raising this point. This is an important issue that we have now addressed. We had previously shown that loss of USP26 degrades SMAD7, therefore leading to stabilization of the TGF- $\beta$  receptor as in the absence of SMAD7 SMURF2 can no longer be recruited to the TGF- $\beta$  receptor complex to ubiquitinate it. The resulting stabilization of the TGF- $\beta$  receptor enhances TGF- $\beta$  activity as evidenced by increased phosphorylation of the SMAD transcription factor SMAD2. We now demonstrate that USP26 also plays a role on SMAD3 activity. Furthermore, we demonstrate that the enhanced activation of these transcription factors leads to an overall enhanced TGF- $\beta$  transcriptional output as we observed increased transcription of TGF- $\beta$  target genes, p21, PAI1, CTGF, and LIF. (**New Figure EV1 C, Figure EV3 E**)

**Reviewer #2:** *The authors showed that USP26 mRNA was upregulated after TGF $\beta$  treatment. They may want to determine the protein levels of USP26 before and after TGF $\beta$  treatment in tumor cells.*

We thank the reviewer for highlighting this important point. Unfortunately, we were not able to identify an antibody that was capable of detecting endogenous levels of USP26.

**Reviewer #2:** *The upregulation of SMAD7 proteins by knock-down of USP26 was only determined in 293FT overexpressing system. It should be determined in tumor cells by detection of the endogenous SMAD7 proteins.*

We understand that the reviewer meant " *The downregulation of SMAD7 proteins by knock-down of*

*USP26 was only determined in 293FT overexpressing system. " We have now demonstrated that knockdown of USP26 diminishes endogenous SMAD7 levels. (New figure 3C, E).*

**Reviewer #2:** *The authors used a reporter system to show the upregulation of TGF $\beta$  signaling by knock-down of USP26. They should provide some evidence of the activation of TGF $\beta$  pathway in tumor cells by checking the downstream targets of TGF $\beta$  pathway.*

We agree and we have now demonstrated that loss of USP26 enhances the transcription of the TGF- $\beta$  target genes SMAD7, CTGF, and LIF to go along with our previous data on SMAD7. (**New Figure EV3 E**)

**Reviewer #2:** *Since the authors used overexpression of USP26 in 293FT cells in a lot of experiments, they may want to present the endogenous and ectopic USP26 protein levels side by side.*

We agree. However, as mentioned we were unable to identify an antibody that was able to detect endogenous levels.

**Reviewer #2:** *Fig 2D, given that the input of USP26 is much less in the pull-down assay of SMAD3, it is possible that SMAD3 had the similar affinity to USP26. The authors may want to clarify it.*

We thank the reviewer for pointing this out. As per the reviewers suggestion we reanalyzed the binding affinity of SMAD3 to USP26. We now demonstrate that SMAD3 appears to bind with a greater affinity to USP26 than SMAD7. We now also demonstrate this interaction endogenously. The fact that USP26 can bind to both SMAD3 and SMAD7 is not surprising. Similarly, USP15 binds to SMAD3 and SMAD7 and recently it has been documented that USP9X can deubiquitinate SMURF1 and SMAD4. Indicating that a number of deubiquitinating enzymes act on multiple nodes of the TGF- $\beta$  pathway to effect overall TGF- $\beta$  output. This point has now been further analyzed in the discussion (**New Figure EV2A, B**)

**Reviewer #2:** *Fig 3 C, D, F, and G used overexpression of multiple plasmid to address the stability of a certain protein, which is questionable. It would be better to determine the stability of the endogenous target proteins either in the stable cell lines or in tumor cell lines.*

We agree. As discussed above we now demonstrate that loss of USP26 degrades endogenous SMAD7 and stabilizes endogenous TGF- $\beta$  receptor I (**New Figure 3C, 3E, 3H**).

**Reviewer #2:** *Fig 3E the actin input was not even, which made the trend of SMAD7 proteins questionable. It is also strange that the TGF $\beta$  stimulation was applied in this assay. Fig 3A and B showed a strong UB signal without TGF $\beta$ , suggesting that TGF $\beta$  stimulation is not required to trigger the degradation of SMAD7.*

We thank the reviewer for their comment. We performed this original experiment in triplicate with cyclohexamide and TGF- $\beta$  adhering to the methodology from other publications analyzing the stability of SMAD7. However, as per the reviewer's suggestions we now observe that USP26 knockdown destabilizes SMAD7 in the absence as well as in the presence of TGF- $\beta$ . We now demonstrate that loss of USP26 enhances SMAD7 degradation in 6 independent figures and feel that in our present manuscript original figure 3E does not significantly add to our argument that loss of USP26 destabilizes SMAD7 and therefore have now excluded this figure.

**Reviewer #2:** *Whereas USP26 knock-down upregulated the ubiquitination of SMAD7, overexpression of USP26 as a DUB to downregulate the ubiquitination of SMAD7 should be presented.*

This was previously shown in our original submission Supplementary figure 2A,B (now labeled figure EV2 E, F).

**Reviewer #2:** *Fig 1D showed that KD of USP26 dramatically upregulated SMAD7 mRNA levels. Is it possible that in tumor cells, lack of USP26 may trigger the upregulation of SMAD7 transcription to compensate for the loss of SMAD7 proteins? The authors may want to do the*



*immunohistochemical analysis of the tumor tissues used in Fig 4 to address the question.*

As part of a negative feedback loop TGF- $\beta$  directly induces the transcription of SMAD7 to downregulate TGF- $\beta$  activity through degradation of the TGF- $\beta$  receptor by the SMAD7:SMURF2 complex. Therefore any factor that affects TGF- $\beta$  receptor stability will likely have an effect on SMAD7 transcription. So increased SMAD7 transcription is not a direct compensatory mechanism for the loss of SMAD7 proteins but rather an effect of overall activation of the TGF- $\beta$  pathway through TGF- $\beta$  receptor stability. Our original glioblastoma samples were purchased through BIOMAX, which unfortunately has discontinued this series of samples. To perform this experiment would require us to perform all of our IHC experiments again at great expense. Nevertheless, we believe we have clearly demonstrated that USP26 is a novel regulator of SMAD7 by showing that loss of USP26 degrades SMAD7 in glioblastoma cell lines suggesting that this would also be true in glioblastoma patient samples.

**Reviewer #2:** *Could the authors perform a combined analysis of TGF $\beta$  and USP26 by using the REMBRANDT database to clarify the importance of USP26 in TGF $\beta$  pathway? They could compare the patient prognosis of TGF $\beta$ High/LowUSP26High/Low groups (4 groups).*

We thank the reviewer for this excellent suggestion. Unfortunately, the REMBRANDT database does not allow for multi-gene analysis. However, we analysed the role of T $\beta$ RI, T $\beta$ RII, T $\beta$ RIII and SMAD7 expression on overall survival. In line with our previous we now demonstrate that high T $\beta$ RI and T $\beta$ RII expression confers poorer overall survival then patients with low T $\beta$ RI and T $\beta$ RII. Similarly, patients with low SMAD7 also exhibited poorer overall survival then patients with high levels of SMAD7. This is consistent with our conclusions that loss of USP26 destabilizes SMAD7 leading to stabilization T $\beta$ RI and overall increased activation of the TGF- $\beta$  pathway (**New Figure EV5B, C, D, E**).

**Reviewer #3:** *Overall, the authors show some interesting results that highlight the potential role of USP26 as a de-ubiquitylase for Smad7 that controls TGF- $\beta$  signaling. The data shown are convincing.*

We thank the reviewer for thinking our results are convincing.

**Reviewer #3:** *- The authors use phosphoSmad2 as read-out of TGF- $\beta$ -induced Smad activation, and then look at transcription targets that are activated by Smad3. Since Smad3 is the major Smad effector for TGF- $\beta$ , some data should show the effect of USP26 on Smad3 activation.*

We agree. We now demonstrate that loss of USP26 also enhances SMAD3 phosphorylation. Curiously, however, SMAD3 phosphorylation was not further induced following the addition of TGF- $\beta$ . (**New Figure EV1 G**)

**Reviewer #3:** *Can the authors show that USP26 associates with endogenous Smad6 or Smad7 (rather than cotransfected, overexpressed proteins)? Is this endogenous interaction ligand-induced?*

We have included new data demonstrating the binding of GFP-USP26 to endogenous SMAD6 and SMAD7. Unfortunately, we were unable to identify an antibody detecting endogenous USP26. Furthermore, we identify that the binding of SMAD7 to USP26 is enhanced following TGF $\beta$ . This observation was not observed for SMAD6 and USP26. (**New Figures 2E, F. Figure EV2C, D**).

**Reviewer #3:** *The authors focus on Smurf2 as an E3 ubiquitin ligase that associates with Smad7. I thought that under the same experimental conditions also the Smurf1 E3 ligase would associate. So, related to Fig. 2F, does USP26 show up in the complex with Smad7 and Smurf1?*

We thank the reviewer for their comment. We have performed these experiments and demonstrate that that like SMURF2, SMURF1 forms a complex with SMAD7 and USP26 (**New Figures EV2G**).

**Reviewer #3:** *Related to the functional link of USP26 with Smurf2-mediated ubiquitylation, what is the effect of increased or decreased USP26 expression on Smad7 levels when Smurf2 expression is downregulated? And what happens to the T $\beta$ RI levels under these circumstances?*

Please see the comments above.

**Reviewer #3:** *Can the authors show that USP26 controls endogenous Smad7 levels?*

Please see the comments above.

**Reviewer #3:** *Does increased or decreased USP26 expression affect the levels of Smad7, when proteasomal degradation is inhibited? This should not be the case, if the authors are right, but such data need to be shown in conjunction with Fig. 3C, D.*

We thank the reviewer for this excellent suggestion. We now demonstrate that the proteasome inhibitor MG132 rescues both ectopically expressed and endogenous SMAD7 levels in cells depleted for USP26. (New Figures 3D, E)

**Reviewer #3:** *Does increased or decreased USP26 expression affect the endogenous Tbr1 levels, and what is the effect on cell surface Tbr1? This is of key importance, but not addressed.*

This has now been completed. (New figures 3H, I).

**Reviewer #3:** *page 3, sentence starting at line 6 from bottom: The authors see Smad7 primarily as a scaffold to recruit E3 ubiquitin ligases, such as Smurf2, to promote Tbr1 degradation. While this statement may serve the authors well to present their findings, most researchers in the field would see Smad7 primarily as an inhibitory Smad that prevents R-Smad activation.*

This has now been corrected.

**Reviewer #3:** *Some incorrect or awkward sentences can be found. Maybe read over it one more time to further polish the text.*

We have edited the text where we felt it may have been confusing and hope that the reviewer is in agreement with our changes.

**Reviewer #3:** *Panels 2A and 2B are results from close to identical experiments with the same results. Only one of these should be shown.*

We thank the reviewer for their suggestion. We had hoped to further enhance our conclusions that loss of USP26 enhances TGF- $\beta$  with multiple non-overlapping hairpins, thereby arguing against an off-target effect. Figure 2B has now been placed in the expanded view section as figure EV1 D.

**Reviewer #3:** *page 7, first sentence of first full paragraph: This statement is an exaggeration.*

This has now been edited.

**Reviewer #3:** *On page 10, halfway: Mark that the Discussion starts there.*

This has been corrected.

2nd Editorial Decision

22 February 2017

Thank you for the submission of your revised manuscript to our editorial offices. As Martina Remold is currently traveling, I am handling your manuscript for the time being. We have now received the reports from the two referees that were asked to re-evaluate your study. Please find them enclosed below.

As you will see, both referees support the publication of your manuscript in EMBO reports. However, both referees have further minor comments that we ask you to address in a final revised version. After cross-commenting with referee #3, we do not think that it is necessary to repeat the experiments indicated by referee #1 using MDA-MB-231 cells (or another tumor cell line) to allow

publication of the manuscript. However, in case you have such data, or can provide this in a timely manner, we would ask you to include these in the final revised manuscript.

Further, I have these editorial requests:

The figures are currently in landscape format. Please submit these as portrait. Please refer to: [http://embopress.org/sites/default/files/EMBOPress\\_Figure\\_Guidelines\\_061115.pdf](http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)

Please provide a title and a legend for Table 1.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

Please add scale bars to all microscopic images.

We noted that large parts of the first paragraph of the introduction are very similar to the same section of a review article you published recently (<http://www.jstor.org/stable/2345678>). I would therefore ask you to rephrase or re-write that part of the manuscript.

We highly appreciate that you submitted the source data for the Western blots. However, could you please split these up and submit one PDF file per figure or EV figure (as these files will then later be linked to the single figures in the online version of the paper).

#### REFEREE REPORTS

##### Referee #1:

In this revised version of the manuscript, the authors satisfactorily addressed most of the concerns and significantly improved the quality of the manuscript.

However, one additional issue should be clarified by the authors: In the new Figure 4G, as equal amount of plasmids were transfected into the cells, why is the GFP-USP26C/S band in Western blot invisible?

In regard to the revision in addressing Reviewer #2's comments: In my opinion, the authors have satisfied most of the reviewer #2's concerns, and consequently enhanced the evidence of molecular mechanism part of the manuscript. However, one major issue, which reviewer #2 has pointed out and I was also concerned, has to be addressed before publication: a lot of functional experiments were still performed only in 293T cells in the revised manuscript, the new Figures 1C, 1E, 1G, 3C, 3E, 3G and 3H should be tested in MDA-MB-231 cells or other tumor cell lines to strengthen the conclusions.

##### Referee #3:

The authors report that the de-ubiquitylase USP26 controls the stability of the inhibitory Smad7, which is known to bring Smurf2 (and perhaps some other E3 ubiquitin ligases) to the type I TGF- $\beta$  receptor (TbRI) and thus promotes TbRI degradation. Consequently, USP26 is seen as a determinant of TGF- $\beta$  signaling. The authors also correlate loss of USP26 with high TGF- $\beta$  activity and poor prognosis in glioblastomas.

This manuscript has been extensively revised in response to the reviewers' comments, and the comments by the reviewers 2 and 3 have been addressed, either in the rebuttal or with new data or in the revised text. I did not evaluate whether the comments of reviewer 1 were addressed. As a result, this is now a much better manuscript with a much better "story".

I have some questions and comments that are minor compared to those of the previous review. I believe that it is worth it that the authors go once more over the text to improve the writing, and, in doing so, address the comments below.

- Important for this manuscript is the statement in paragraph 2 of page 4, dealing with the relative roles of Lys48 and Lys63. For this statement the authors refer to reference 16, but I do not see that information in reference 16, which, incidentally, is a review that does not deal with TGF- $\beta$  signaling. So, where does this information come from?
- At several points in the Discussion, statements are made that would benefit from having a reference attached to it.
- "data" is plural. So, "this data suggests" should be "these data suggest". This is relevant for a number of sentences in the text.
- The authors exceedingly use "as expected" in their Results section, but not everything is necessarily "as expected".
- The "official" recommendation is to use "ubiquitylation" rather than "ubiquitination". This is an issue for the entire manuscript.
- The Introduction and Discussion could be shorted with 20% without loss. They tend to go on...
- Fig. 3A is not convincing to me and does not show clearly what is concluded from it. Also, that sentence (page 7, lines 4 and 3 from the bottom) is grammatically not clear.
- Fig. 3I: I am surprised that cell surface TbRI shows up as such a tight band, since it is normally glycosylated.

2nd Revision - authors' response

01 March 2017

Thank you very much for your thorough revision of our manuscript entitled " USP26 regulates TGF- $\beta$  signaling by deubiquitinating and stabilizing SMAD7 " REF: EMBOR-2016-43270V2. I am happy to say that we have addressed all the relevant comments put forward by you and the reviewers. These revisions are highlighted in our point to point responses. We believe this final edited version now meets the criteria to warrant publication in EMBO Reports.

-----  
Responses to reviewer's comments:

We thank the editor and the reviewers for their thoughtful comments and thorough revision of the manuscript. We have addressed the majority of the concerns and comments raised by the reviewers and edited the manuscript accordingly.

Editor: The figures are currently in landscape format. Please submit these as portrait.

*Response: This has now been corrected.*

Editor: Please provide a title and a legend for Table 1.

*Response: This has now been included.*

Editor: Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

*Response: This has now been included where applicable*

Editor: Please add scale bars to all microscopic images.

*Response: These have now been included.*

Editor: We noted that large parts of the first paragraph of the introduction are very similar to the same section of a review article you published recently (<http://www.jstor.org/stable/2345678>). I would therefore ask you to rephrase or re-write that part of the manuscript.

*Response: Apologies for the overlap between the two articles. We have now formatted the first paragraph of the introduction.*

Editor: We highly appreciate that you submitted the source data for the Western blots. However, could you please split these up and submit one PDF file per figure or EV figure (as these files will then later be linked to the single figures in the online version of the paper).

*Response: This has now been done.*

-----  
 Referee #1: In the new Figure 4G, as equal amount of plasmids were transfected into the cells, why is the GFP-USP26C/S band in Western bolt invisible?

*Response: We thank the reviewer for their comment. We have noticed that USP26 DUB dead is significantly less stable then WT USP26. (Fig. 2B, 3A, 3G). Our results in figure 4 are consistent with these observations. However, we have now included a higher exposure of this figure to better demonstrate expression of USP26 DD in this experiment.*

Referee #1: In regard to the revision in addressing Reviewer #2's comments: In my opinion, the authors have satisfied most of the reviewer #2's concerns, and consequently enhanced the evidence of molecular mechanism part of the manuscript. However, one major issue, which reviewer #2 has pointed out and I was also concerned, has to be addressed before publication: a lot of functional experiments were still performed only in 293T cells in the revised manuscript, the new Figures 1C, 1E, 1G, 3C, 3E, 3G and 3H should be tested in MDA-MB-231 cells or other tumor cell lines to strengthen the conclusions.

*Response: We thank the reviewer for their comments. In relation to figures 1C, 1E, and 1G this work was previously demonstrated in MCF7, MDA-MB-231, T47D, CAL51, U373, A172, and the patient derived cell line (Figure EV4). Furthermore, we clearly demonstrate that pSMAD2 is enhanced in the breast cancer cell lines MDA-MB-231 and MCF7 depleted for USP26, suggesting that a similar mechanism must exist in for enhanced SMAD phosphorylation in both 293T cells and these breast cancer cell lines.*

Referee #3: - Important for this manuscript is the statement in paragraph 2 of page 4, dealing with the relative roles of Lys48 and Lys63. For this statement the authors refer to reference 16, but I do not see that information in reference 16, which, incidentally, is a review that does not deal with TGF- $\beta$  signaling. So, where does this information come from?

*Response: In general, we like to thank the reviewer for their thorough review of our work their suggestions have vastly improved our manuscript. In regards to their comment, we have now changed the reference. However, it is important to note that our comments regarding ubiquitin chain topology and their role is regulating substrate function is a general feature of ubiquitin mediated substrate targeting which is not only exclusive to the proteins in the TGFB pathway but to all proteins in the cell.*

Referee #3: At several points in the Discussion, statements are made that would benefit from having a reference attached to it.

*Response: This has now been edited accordingly.*

Referee #3: "data" is plural. So, "this data suggests" should be "these data suggest". This is relevant for a number of sentences in the text.

*Response: Thank you. These grammatical errors have now corrected.*

Referee #3: - The authors exceedingly use "as expected" in their Results section, but not everything is necessarily "as expected".

*Response: Where appropriate we have minimized our expectations.*

Referee #3: - The "official" recommendation is to use "ubiquitylation" rather than "ubiquitination". This is an issue for the entire manuscript.

*Response: We have made these recommended changes in the text.*

Referee #3: - The Introduction and Discussion could be shorted with 20% without loss. They tend to go on...

*Response: Thank you we have attempted to shorten our introduction and discussion while retaining the most relevant details for our manuscript. We believe our editions have greatly improved the readability of our manuscript.*

Referee #3: - Fig. 3A is not convincing to me and does not show clearly what is concluded from it. Also, that sentence (page 7, lines 4 and 3 from the bottom) is grammatically not clear.

*Response: We agree and have now quantified the data to more effectively demonstrate our conclusions. We have also now edited the text as indicated by the reviewer.*

Referee #3: Fig. 3I: I am surprised that cell surface TBRI shows up as such a tight band, since it is normally glycosylated.

*Response: We thank the reviewer for this excellent comment. Although it is expected to see glycosylated isoforms for TBRI they are very difficult to detect. This may be because the proportion of glycosylated TBRI is relatively small compared to non-glycosylated isoforms or that the half-life of glycosylated TBRI is short. On the other glycosylated TBR II is readily detectable. Similar observations to our own were observed in Fig. 3H. Zhang et al. Molecular Cell 51, 559-572 September 12, 2013.*

---

3rd Editorial Decision

07 March 2017

Thank you for submitting your revised manuscript to EMBO reports. Browsing through the manuscript I found some minor issues that need to be corrected. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once these few minor corrections have been addressed, as follows.

- Please add a scale bar to the images in Fig. EV5
- Please complete the information on data quantification for Fig. 1A (SD, n), 4B (n=1?), and EV1A.
- I have not looked at all the source data, but I happened to notice a mistake in the source data file for Fig. EV2G. The blots are labeled with 'anti-Myc'. If I am not mistaken this should be 'anti-HA'.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue. Thank you for your contribution to EMBO reports.

---

3rd Revision - authors' response

08 March 2017

The authors made the requested changes and submitted the final version of their manuscript.

---

4th Editorial Decision

08 March 2017

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

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Pieter Eichhorn  
 Journal Submitted to: Embo Reports  
 Manuscript Number: EMBOR-2016-43270V2

## Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

## USEFUL LINKS FOR COMPLETING THIS FORM

<a href="http://www.antibodypedia.com">http://www.antibodypedia.com</a>	Antibodypedia
<a href="http://1degreebio.org">http://1degreebio.org</a>	1DegreeBio
<a href="http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo">http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo</a>	ARRIVE Guidelines
<a href="http://grants.nih.gov/grants/olaw/olaw.htm">http://grants.nih.gov/grants/olaw/olaw.htm</a>	NIH Guidelines in animal use
<a href="http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm">http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm</a>	MRC Guidelines on animal use
<a href="http://ClinicalTrials.gov">http://ClinicalTrials.gov</a>	Clinical Trial registration
<a href="http://www.consort-statement.org">http://www.consort-statement.org</a>	CONSORT Flow Diagram
<a href="http://www.consort-statement.org/checklists/view/32-consort/66-title">http://www.consort-statement.org/checklists/view/32-consort/66-title</a>	CONSORT Check List
<a href="http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur">http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur</a>	REMARK Reporting Guidelines (marker prognostic studies)
<a href="http://datadrvgd.org">http://datadrvgd.org</a>	Dryad
<a href="http://figshare.com">http://figshare.com</a>	Figshare
<a href="http://www.ncbi.nlm.nih.gov/gap">http://www.ncbi.nlm.nih.gov/gap</a>	dbGAP
<a href="http://www.ebi.ac.uk/ega">http://www.ebi.ac.uk/ega</a>	EGA
<a href="http://biomodels.net/">http://biomodels.net/</a>	Biomodels Database
<a href="http://biomodels.net/miriam/">http://biomodels.net/miriam/</a>	MIRIAM Guidelines
<a href="http://jrib.biochem.sun.ac.za">http://jrib.biochem.sun.ac.za</a>	JWS Online
<a href="http://oba.od.nih.gov/biossecurity/biossecurity_documents.html">http://oba.od.nih.gov/biossecurity/biossecurity_documents.html</a>	Biossecurity Documents from NIH
<a href="http://www.selectagents.gov/">http://www.selectagents.gov/</a>	List of Select Agents

## 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?	The sample size was chosen and evaluated according to previously established protocols warranted statistically significant in our field of study.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A. All samples were included in our final criteria.
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.	All samples were treated at the same time and processed in the manner to ensure that no bias was being observed.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	All proteinsamples were treated in a non biased fashion. For evaluation of IHC stains a trained pathologists assessed the results in a double blind fashion.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes they are applicable.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All statistical analysis of all replicates was determined -/+ standard deviation of three independent experiments. P Value was determined by two tailed T test.
Is there an estimate of variation within each group of data?	where appropriate all experiments were analysed by standard deviation of three independent experiments.
Is the variance similar between the groups that are being statistically compared?	Variance between groups is statistically significant.

## 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).	N/A (5MAD [1108], and 11 smad2 [1103], wnt from cell signaling. Anti smad4 (sc-7966), anti smad7 (sc-7004), anti HA (Y-11), anti gfp (sc-57592), anti myc (sc-795), and brr1 (sc-358) were from santa cruz
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, MCF7, MDA-MB-231, U373, A172, T47D, and CAL51 were purchased from ATCC. PCTC was a kind gift from Joan Seoane. All cell lines were tested for mycoplasma contamination.

\* For all hyperlinks, please see the table at the top right of the document

## D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

## E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Samples were purchased from Biomax and protocols approved by NUS-IRB.
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 accession codes for deposited data. See author guidelines, under '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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.  Examples: <b>Primary Data</b> Weitzme KX, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 <b>Referenced Data</b> Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CRA/5 of TR. Protein Data Bank 4Q26. AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PX0000208	N/A
22. 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 Biomedels (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

23. 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
---	-----