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USP4 inhibits SMAD4 monoubiquitination and promotes activin and BMP signaling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 September 2016

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision but I have now received the comments back from the three referees.

As you can see from the comments below, referees #1 and 2 both find the analysis insightful. Referee #3 is not so persuaded that the novel insight provided is sufficient to consider publication here. Having read the manuscript, I am in agreement with referees # 1 and 2 and find that the analysis provides important new insight. I would therefore like to invite you to submit a suitable revised version - please note that major revisions are needed. The concerns raised are clearly outlined below and I will not repeat them here. One issue that I want to highlight is that it would be good to sort out the contributions of USP9x and Ectodermin in your system - ref#1 point #1. This issue remains an open question and adding clarity on this aspect would be important.

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

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Let me know if we need to discuss anything further.

REFEREE REPORTS

Referee #1:

MS #: EMBOJ-2016-95372
MS TITLE: USP4 controls activin and BMP signaling in ES cells and zebrafish morphogenesis by inhibiting SMAD4 monoubiquitination
AUTHORS: Zhou et al.

Activation of the TGF-β signaling pathway plays a critical role in many biological process, including embryonic development, EMT and a number of human diseases, including cancer. A number of feedback mechanisms have evolved to modulate the strength and duration of TGF-β signaling. These mechanisms include association with the transcriptional inhibitor c-Ski and the reversible ubiquitination of the common TGF-β effector molecule, Smad4. A previous study showed that Smad4 is modified by the addition of a single ubiquitin on lysine 519 by the E3 ligase, Ectodermin. This process is reversed by the de-ubiquitinating enzyme (DUB), USP9x. Monoubiquitination inhibits Smad4 function, whereas removing this ubiquitin restores Smad4 function.

This is a complex paper, with many moving parts, but the data greatly extend our understanding of how ubiquination modulates the response to TGF-β signals in several important ways. The main conclusions from the paper are:

1) The DUB USP4 acts directly on Smad4 to remove the mono-ubiquitination of Smad4 on Lys 519, which potentiates the response to BMP and Activin signals in cell culture, MEFs, ES cells and in zebrafish embryos. USP4 does not modify the R-Smads.
2) USP4 binds to monoubiquinated Smad4 and recruits other DUBs (USP 11 and USP15) into a deubuiquinating complex.
3) Smurf2 catalyzes the monoubiquitination of Smad4, which dampens prevents its ability to bind phospho-R-Smads and dampens its activity in cells. This result is surprising given the documented role of Ectodermin.
4) In response to TGF-β signals, the TGF-β antagonist c-Ski binds to phosphorylated R-Smads and to Smurf2, resulting in the ubiquitination of c-Ski and its degradation.
5) The Akt Ser/Thr kinase controls USP4 function in mES cells by phosphorylating it on SER 445. Phospho-USP4 is activated and can , permitting it to target Smad4 for deubiquitination.
6) USP4 is required in zebrafish embryos to repress BMP and Activin/Nodal target genes, and to control the cell movements of epiboly.

Most of the experiments are well-designed and show the proper controls. While many of the conclusions rely on analysis of gain-of-function experiments, which could be misleading, they are complimented by corresponding loss-of-function studies. The effectiveness of the shRNAs is shown by the appropriate Western Blots. In short, this is an important study that significantly extends our knowledge of how TGF-β signaling is regulated. By and large, the data are convincing, but there are a few questions that need to be addressed prior to publication.

Major Points:
1) It is unclear how USP9x and Ectodermin fit into the model presented by the authors. The authors state in the discussion that "cellular context" may be important, but it appears that at least some of the experiments presented here were performed in the same cell lines as reported by Dupont (2009). Could the two proteins be acting redundantly?

2) USP15 (along with USP5) was previously implicated as a regulator of BMP signaling in

zebrafish, but USP15 morphants display a ventralized phenotype consistent with excess BMP signaling (Tse et al., 2009). How does can this result be reconciled with the data presented here that USP15 de-ubiquinates Smad4 in a USP4 dependent manner in MEFs (Fig. 3M)? If this were correct, one would predict that USP15 loss-of-function embryos would have more inactive, monoubiquinated Smad4 and be less responsive to BMP signals, not more responsive.

3) Tse et al. 2009 also report the phenotype of USP4 morphants, though not in great detail. This work should be cited and it would be helpful to know how the phenotype of the morphants in this work compare with the previously published phenotype. In fact, a live image of the USP4 morphant phenotype at 24 hpf would be very helpful. How does this compare to the defects seen in bmp2/swirl or Nodal mutant embryos?

4) None of the MO sequences used are indicated. Are these translation blocking MOs, or splice blocking? The standard in the field is that the phenotype should be replicated by two MOs of unrelated sequence. In addition, experiments should be presented indicating the efficiency of the MO knockdown, and showing the ability of a specific mRNA to rescue the morphant.

5) The epiboly delay shown in Fig. S7 is quite mild. In fact, the images at 7 hpf show very little delay (the top image of a single embryo is not at the correct angle for comparison with the lower embryo). Epiboly delay is a common MO artifact, and is often seen at high MO doses and may not be observed in the control MO. What MO dose is used in these experiments? Is the delay rescued by mRNA injection? Also, It looks like the embryonic shield does not form in the morphants. Is this the case?

6) How does USP4 effect expression of direct targets of the BMP and Nodal pathways. Expression of Bmp2 in Fig. 1J is good, since BMP2 autoregulates, but another direct target would be helpful (such as Tbox6). Expression of squint and cyclops would also be useful in this context. Does USP4 overexpression lead to an upregulation of these target genes, as predicted?

7) There are so many panels in each Figure, that the data can be difficult for the reader to see. This problem will be exacerbated on the published page. The zebrafish embryo images shown in Figs. 1I, J, and Fig 7A are especially tiny. I understand the desire to show that the embryos are not cherry picked, but a single representative image should be selected for each stain (WT and Control) and shown in each panel. Leave the multiple embryo panels for the supplement, if necessary.

8) Fig. 5D is very confusing. Which part of the gel is the supernatant fraction and which was the pellet. This experiment needs to be described better in the text, and the figure itself should be clarified.

9) Does the USP4-CS mutant act like a dominant negative molecule?

Minor Points:

1) Fig. 6D and Fig. 6E show smaller colony sizes in USP4 knockdown mES cells than in controls. But on page 15, the authors claim that this is due to reduced proliferation. There is no evidence to support this conclusion.
2) p8. 'a great portion of Smad4 might be monoubiquinated'. This is a vague statement. Can this be quantified?
3) p14. 'depletion led to a much lower activin signal than in wild type mECs... (Fig. 6B)" Again, can this vague statement be quantified?

There are several typos throughout the manuscript, some of which are confusing to the reader. I list below some of the typos I marked:

p3. "removal from TGF-β family components" should be "removal OF TGF-β"
p7. 'unpon' should be 'upon'
p8. 'monoubiquinated SMAD4 avidly interacts with SMAD4" should read 'monoubiquinated SMAD4 avidly interacts with USP4'

Referee #2:

This group previously published that USP4 functions to deubiquitinate the TGFbeta type I receptor and thus acts to promote TGFbeta signaling.
In contrast, in this study it is reported that USP4 does not modulate activin/BMP type I receptors rather that USP4 removes mono-ubiquitin on Smad4 and thereby promotes activin/BMP signaling. They go on to show that Smurf2 (which is known to mediate ubiquitination and degradation of several TGFbeta family components), but not Smurf1 or TRIM33, is responsible for Smad4 mono-ubiquitination. A model is presented in which USP4 de-monoubiquitinates Smad4, which promotes Smad complex binding to DNA elements and that this is followed by recruitment of Smurf2 that then monoubiquitnates Smad4 to terminate transcription. Actually, this model is remarkably similar to that proposed by Dupont et al except that the DUB and E3 ligases reported as being responsible in the Dupont study were USP9X and TRIM33, respectively. There is some controversy in the literature as to the precise role of TRIM33 in TGFbeta signaling and this manuscript provides further evidence that our understanding of TRIM33 function in the pathway may require further consideration.

The data presented in this paper in cultured cell lines and mESC supports the conclusion that USP4 enhances activin/BMP signalling. Biochemical data is also presented that is consistent with the idea that USP4 is capable of removing monoubiquitin from Smad4 (with the caveat that additional controls should be included as described below). However, the study would have been enhanced with a more direct demonstration that this is the mechanism whereby USP4 acts to regulate activin/BMP signaling, perhaps through some kind of rescue with a mutant variant of Smad4. Without this, a more cautious title and abstract would be more appropriate.

Finally, there are some results presented which add little to the overall study and rather serve as a distraction, such as data showing binding of other USPs to USP4 and the role of AKT-mediated phosphorylation of USP4 in mESCs. In this reviewer's opinion, these should be removed.

Specific comments:

Fig. 1B. Cell line should be mentioned in the text here rather than later in panel E.

Fig. 1C. Knockdown efficiency should be shown.

Fig. 1H-K. The authors neglected to indicate in the text that these panels refer to zebrafish embryos. The USP4 knockdown efficiency needs to be included.

Page 7. "Indeed, unlike its activity towards TGF-β type I receptor (Zhang et al, 2012b), USP4 did not act as a DUB for activin/BMP type I receptors (data not shown)." Can the authors provide any explanation for this result?

Fig. 2F-J. There is a vertical line marked in all the gel blots that separate the control from the treated samples. Although it is not indicated in the legend, this type of line is typically used to indicate that the blot was spliced. It is extremely concerning that this key control (ie untreated sample) has been spliced in all experiments showing that USP4 can de-ubiquitinate Smad4.

Fig. 2I. The paper centers on the mono-ubiquitination of Smad4. Thus, a better indication that the HA band is indeed mono-ubiquitinated Smad4 would be useful (ie reblot the HA blot with Flag or include position of markers and show both blots side by side to indicate relative migration. Also, what is the evidence that this is mono-ubiquitin as opposed to say 2 ubiquitins on Smad4?

Fig. 3C-G. To be sure it is ubiquitinated Smads that are being visualized by HA blotting and not simply that the antibody is recognizing total Smad4, a control of cells transfected with SMAD4 but not HA-ubiquitin should be included.

Fig. 3D. The importance of testing the mutant forms of ubiquitin was not clear. Perhaps this might be best as part of the supplementary data.

The data regarding USP4 stabilization by interacting with USP11/15 adds little to the paper and

could be removed.

Fig. 4A-B. Do the authors have any pull-down experiments that include a negative control to ensure non-ubiquitinated Smad4 is not binding non-specifically?

Fig. 4A. The legend is not written correctly based on what is in the text. "HEK293T cells transfected with SMAD2 along with SMAD4 or SMAD4-1xUb".

Fig. 4D. The text, legends and panel labels are not sufficient to discern what is being shown. What cells were used? Is this endogenous proteins or transfected cells? If transfected, then the ns control is not useful without showing totals.

Fig. 6D and E. The requirements of mES cells for activin and/or BMP are rather complex, so the authors need to support their statement (based on literature if available) on how smaller colony sizes and slower proliferation for USP4 depleted cells represents evidence of a defective activin/BMP response.

Page 15 and associated panels relating to AKT. The examination of the putative role of AKT-mediated phosphorylation of USP4 on Smad monoubiquitination and activin/BMP signalling is a side issue and could more appropriately form the basis of a more detailed follow-up study.

Other comments:
Al-Salihi et al, Agricola et al and many other references are missing the year.

Top of page 4, citation list has an extra set of brackets. Same issue occurs elsewhere.

In the introduction, the paragraph describing the role of c-Ski in Smad complexes and transcription was particularly unclear, ie two particularly problematic sentences in were: "C-SKI inactivates proper R-SMAD-SMAD4 complex formation" and "Inactive SMAD binding to promoters of target genes is promoted by c-SKI"

What is the origin of the ARE reporter used in the screen? Presumably, this is not the similarly named Foxh1-dependent reporter (as Foxh1 is not expressed in HEK293T cells).

SMAD typo (SMDA) on 3rd last line of page 5, Fig. 4C and in other places

There are missing periods and spaces throughout text.

Page 8. A correction is needed of: "SMAD4 avidly interacts with SMAD4"

Methods: A description of how mono-ubiquitinated Smad4 was purified (a key reagent) is missing. The relevant section in the methods erroneously refers to mono-ubiquitinated TBRI purification, but even so no details are provided.

Page 13, line 1: 'results' is misspelled

Page 14, line 3 and line 6. The authors refer to 'its' degradation. Since there are several proteins named in the sentences, it would significantly improve clarity if the specific protein that was degraded was actually named (ie c-SKI).

Page 14: The authors need to indicate where the data can be found for this statement: "our observed staining of c-SKI-trapped SMAD4 in the nucleus"

Page 14. In this sentence: "USP4 depletion led to a much lower activin signal than in wild type mECs, as shown by a reduction in SMAD2-SMAD4 complex formation" the phrase " a much lower activin signal" is unclear/vague and should be removed.

Figure 7. The presentation of the results of the zebrafish data would be enhanced if the authors would clarify how the observed developmental effects upon loss of USP4 relate to expected changes in activin and/or BMP signalling.

For many experiments, the authors neglected to indicate the number of times the experiments were reproduced.

Referee #3:

In this paper the authors show that the deubiquitin enzyme USP4 interacts with the co-Smad, Smad4, and catalysis its deubiquitination. Monoubiquitination of Smad4 is found to be induced by ligand-stimulated R-Smad-Smad4-Smurf2 complex. My major concern about this manuscript, aside from the fact that it is very poorly written, is the novelty. Monoubiquitnation of Smad4 has been reported to be a way of inactivating this co-Smad following complex formation with R-Smads. DUBs have also been reported to stimulate TGF-beta/BMP signaling by allowing R-Smads and Smad4 to recycle back into signaling events. The link between TGF-beta signaling and USP4 was also already known as previous work demonstrated that USP4 acts as a de-ubiqutinase for the type I TGF-beta receptor. As such, and considering that the manuscript is close to a draft, I would not recommend its publication in a high quality journal like EMBO J. The experimental work appears to be well done but it is presented in such a poor way that no coherent story can be derived from the manuscript.

When re-submitting, the authors would do themselves a favor by proof-reading their manuscript before sending it for publication. The peer-review process is here to ensure the scientific quality of the work and not to correct numerous writing mistakes. Examples of such mistakes can be found below:
- The introduction is poorly written in a telegraphic style. References are wrongly called (multiple et al without the date of publication).
- Page 8 "monoubiquitinated SMAD4 avidly interacts with SMAD4").

1st Revision - authors' response 18 January 2017

Referee #1
Specific comments:

Major Points:
1) It is unclear how USP9x and Ectodermin fit into the model presented by the authors. The authors state in the discussion that "cellular context" may be important, but it appears that at least some of the experiments presented here were performed in the same cell lines as reported by Dupont (2009). Could the two proteins be acting redundantly?

Response: In line with Piccolo and coworkers (Dupont et al, 2009), we found that SMAD4 is mono-ubiquitinated on K519 that disrupts its complex formation with activated R-SMADs and interferes with DNA binding. However, we could not see any effect of ectopically expressed V5-USP9x on SMAD4 mono-ubiquitination. The result is shown below (Figure A). We have included a remark about this in the paper.

Furthermore, we compared USP4 with USP9x in the activin-induced transcriptional response (new Supplementary Figure S1C). While USP4 potently activated ARE-Luc reporter, USP9x showed no significant effect. Importantly, this response was impaired by sh-USP4 targeting the 3'UTR USP4 sequence, which could be nicely rescued by USP4 but not by USP9x. These results demonstrated that USP4 is not likely acting redundantly with USP9x. We included a statement on these findings in the text.

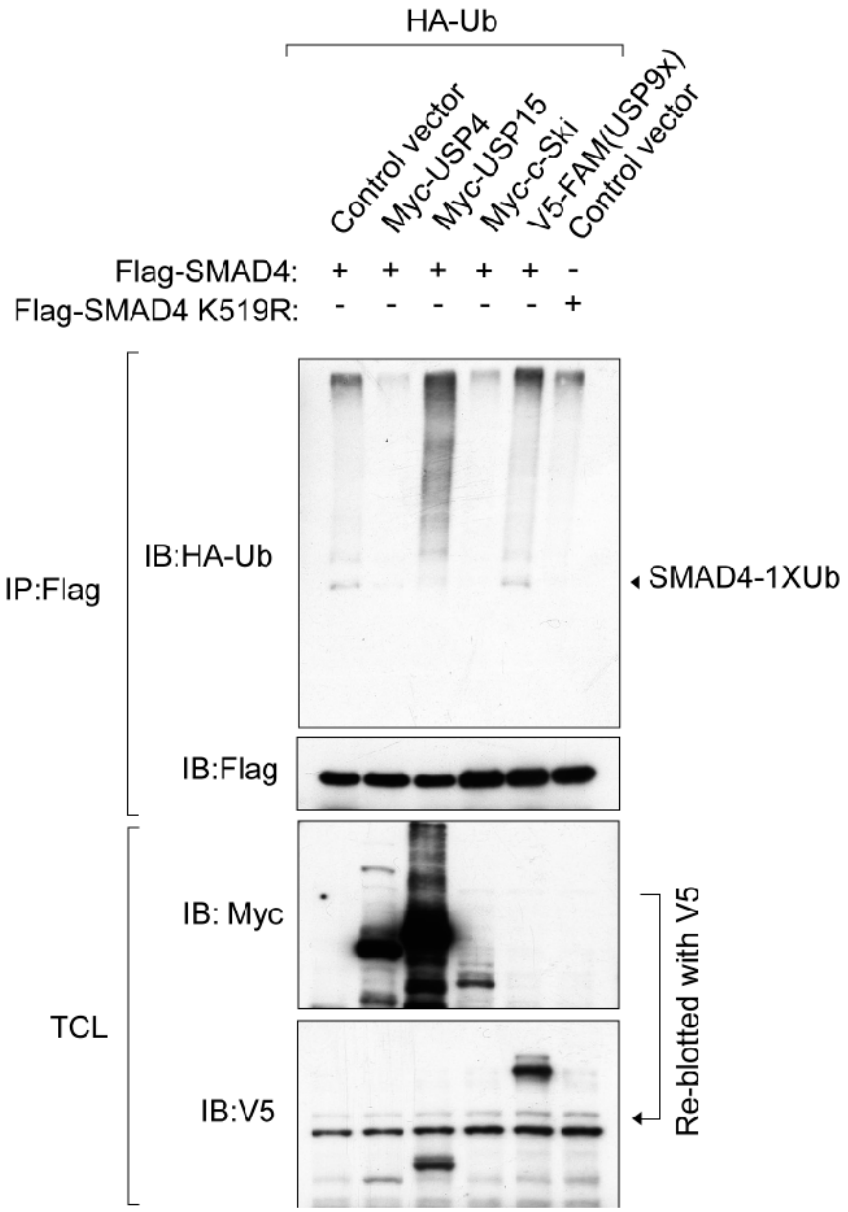


Figure A
Immunoblot (IB) analysis of total cell lysate (TLC) and immunoprecipiates derived from HEK293T cells transfected with Flag-SMAD4 and control vector, Myc-USP4, Myc-USP15, Myc-c-SKI or V5-USP9x. Position of monoubiquitinated SMAD4 is indicated. In contrast to USP4 (and USP15 and c-SKI), USP9x does not affect mono-ubiquitination of SMAD4.

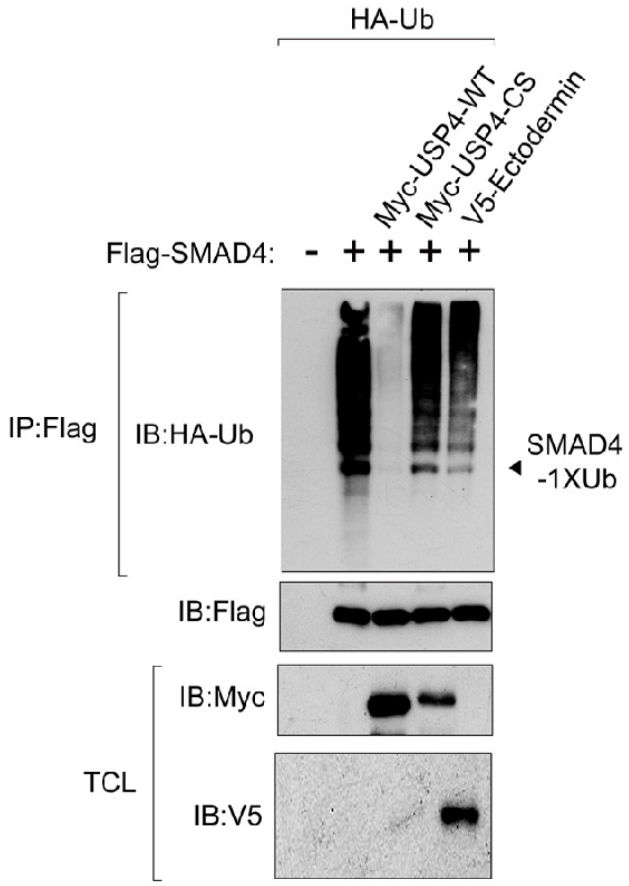


Figure B

TRIM33/Ectodermin did not affect SMAD4 monoubiquitination under the conditions of transient co-transfection with SMAD4. HEK293T cells that stably express HA-Ub were transfected with expression plasmids for Flag-SMAD4, Myc-USP4-WT, Myc-USP4-CS or V5-Ectodermin as indicated. The cell lysates were harvested for ubiquitination assay. The position of monoubiquitinated SMAD4 is indicated

We observed no effect of TRIM33/Ectodermin on the level of monoubiquitination of SMAD4 by coexpressing TRIM33/Ectodermin and SMAD4 in HEK293T cells (Figure B). This is not in agreement with the publication by Dupont and co-workers (Dupont et al. 2005) who demonstrated that TRIM33/Ectodermin interacts with SMAD4 and induces ubiquitination of SMAD4. Our negative result made us less interesting to pursue the role of TRIM33/Ectodermin in SMAD4 monoubiquitination, in particular as we were able to get a positive result with SMURF2. In addition, we found that SMAD4 monoubiquitination is strongly potentiated by TGF-b/R-SMAD activation. This made us to hypothesize that the E3 ligase for SMAD4 monoubiquitination is recruited to SMAD4 in a TGF-b dependent manner. This prompted us look at the potential role of SMURFs in this process; they are capable to interact with R-SMADs but not SMAD4, and R-SMAD and SMAD4 form a complex upon TGF-b stimulation. In experiments we could show that SMURF2, but not SMURF1, is recruited to SMAD4 in an activated R-SMAD-dependent manner. In another later publication, Dupont and coworkers report that TRIM33/Ectodermin induces the mono-ubiquitination of SMAD4 and that this is potentiated by TGF-b and SMAD2 activation (Dupont et al, 2009). The absence of ligand addition and cotransfection with SMAD2 in our experimental set up (Figure B) may have been the reason why we did not detect SMAD4 monoubiquitination activity of TRIM33/Ectodermin.

Our proposed model and the model of Dupont and co-workers (Dupont et al, 2009) are conceptually similar but with involvement of different E3 ligases. The TRIM33/Ectodermin and SMURF2 are RING versus HECT domain containing E3 ligases, respectively. Also TRIM33/Ectodermin has a bromodomain and contact of this domain with histones is needed for E3 ubiquitin ligase activity (Agricola et al, 2011). This property is not shared with SMURF2. Thus, both molecules are likely to act in different subcellular compartments and are subject to different regulation. We have included a discussion about this in our revised manuscript.

2) USP15 (along with USP5) was previously implicated as a regulator of BMP signaling in zebrafish, but USP15 morphants display a ventralized phenotype consistent with excess BMP signaling (Tse et al., 2009). How does can this result be reconciled with the data presented here that USP15 de-ubiquinates Smad4 in a USP4 dependent manner in MEFs (Fig. 3M)? If this were correct, one would predict that USP15 loss-of-function embryos would have more inactive, monoubiquinated Smad4 and be less responsive to BMP signals, not more responsive.

Response:
usp15 morphants in zebrafish have been reported to display a weakly dorsalized (or reduced ventralizing) phenotype indicative of a decreased BMP signaling (Tse et al, 2009). Moreover, this result was further consolidated in a more recent paper by the same group (Tse et al, 2013) in which the interruption of BMP4 signaling by double knockdown of *usp15* and *otud4* reduced the ventralizing effects that were induced by the overexpression of *tsc22d3*. These data are fully consistent with our observed *usp15* phenotype; similar as was reported by Tse et al, our *usp15* morphants showed a C2 type dorsalized phenotype (see results below).

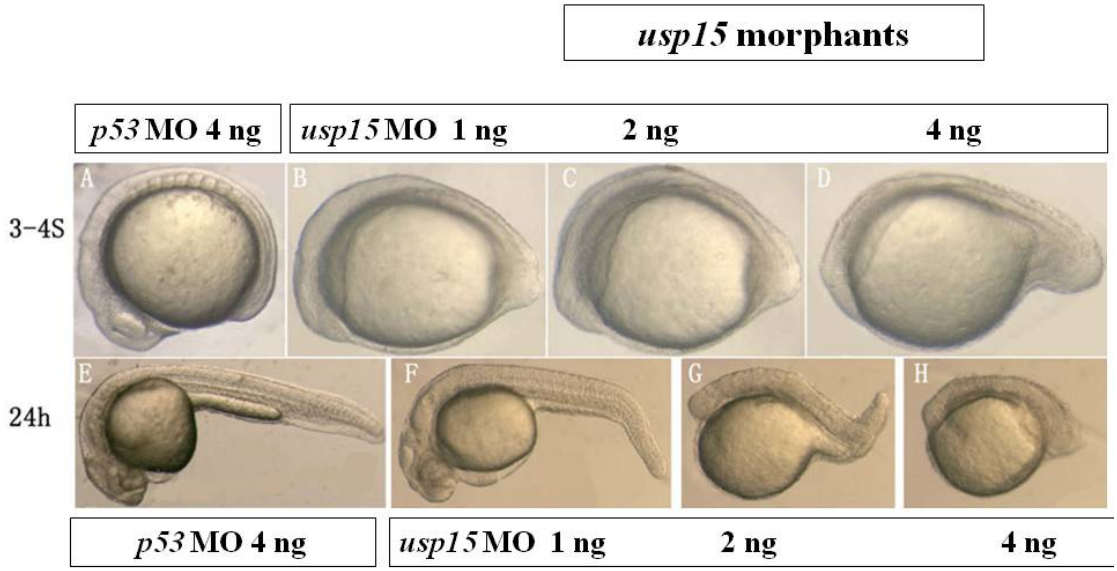


Figure C
Single-cell embryos were injected by control *p53* MO, *usp15* MO or their combination. The developmental stages included 3-4 somites stage and 24 hpf. The MO dose that was injected is indicated.

Our results above are also in line with previous reports on USP15 function in cultured mammalian cells and *Xenopus* embryos. Piccolo and co-workers reported that USP15 functions as a DUB for BMP R-SMAD1 and SMAD5 by opposing mono-ubiquitination that prevents promoter recognition (Inui et al., 2011). Morpholinos targeting USP15 and USP4 in *Xenopus* were reported by them to inhibit the expression of BMP target gene *Sizzled*. Sapkota and co-workers found that USP15 deubiquitinates and stabilizes BMP type I receptor ALK3. Morpholino-mediated knockdown of USP15 in *Xenopus* inhibited the expression of BMP target gene *Vent1* (Herhaus et al., 2014).

We found yet another mechanism by which USP15 promotes BMP signaling. USP4 and USP15 interact and functionally cooperate with each other. USP15 removes monoubiquitin from SMAD4 in a USP4-dependent manner. Consistently, we observed that USP15 in cultured cells potentiates the BMP/SMAD-induced BRE-Luc transcriptional response in a deubiquitinating enzyme dependent manner.

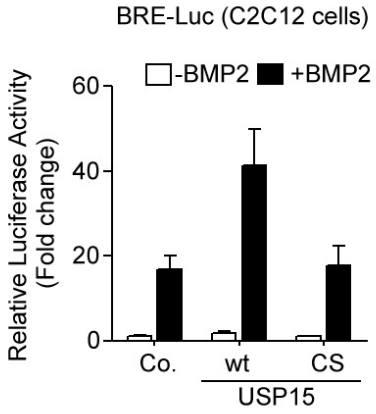


Figure D
Effect of USP15 wild type (USP15-WT) and the USP15 mutant that lacked deubiquitinating enzyme activity (USP15-CS) on BMP2-induced BRE-luc transcriptional luciferase reporter activity in C2C12 myoblasts. Co, empty vector.

3) Tse et al. 2009 also report the phenotype of USP4 morphants, though not in great detail. This work should be cited and it would be helpful to know how the phenotype of the morphants in this work compare with the previously published phenotype. In fact, a live image of the USP4 morphant phenotype at 24 hpf would be very helpful. How does this compare to the defects seen in bmp2/swirl or Nodal mutant embryos?

Response: Tse et al., 2009 report that USP4 morphant falls in group II, which is characterized by a decreased in neuronal *huC* marker expression. We have included a citation to the Tse et al paper regarding the USP4 morphant phenotype.

Comparison of *usp4* morphant and *bmp2/swirl* mutant revealed substantial overlap in phenotype in the early stage of gastrulation, like the expanded DV axis and the epiboly delay (see Figure F below). However, some significant differences between *usp4* morphant and *bmp2/swirl* mutant were also observed. In the swirl mutant embryos, dorsal structures such as notochord and somites are expanded, while ventral structures such as blood and nephrons are missing (Kishimoto et al, 1997).

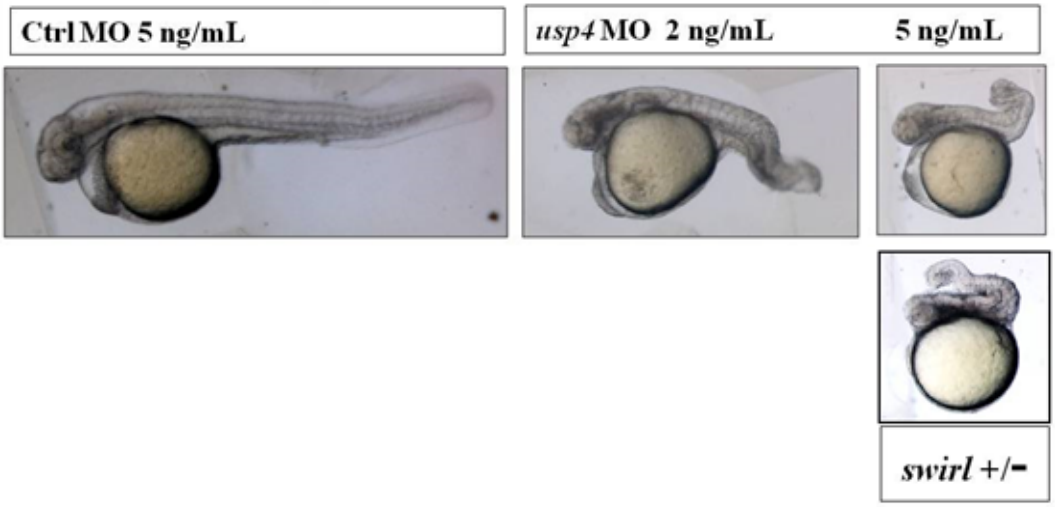


Figure E
The phenotypes of *usp4* morphants and *swirl* mutant embryos. The swirl heterozygous embryos displayed a more dorsalized phenotype.

4) None of the MO sequences used are indicated. Are these translation blocking MOs, or splice blocking? The standard in the field is that the phenotype should be replicated by two MOs of

unrelated sequence. In addition, experiments should be presented indicating the efficiency of the MO knockdown, and showing the ability of a specific mRNA to rescue the morphant.

Response: The specificity and efficiency of *usp4* MO2 have been described in our previous publications (Zhang et al, 2012; Zhou et al, 2012). These two references are included in the revised manuscript.

We have used a *usp4* splicing blocker MO (MO2): 5'-TGAACATCTTTGTATACCTGGAGGG-3' and MO2 mismatch control: 5'-TCAAGATGTTTCTATACCTGGAGGG-3'. There is 84% of sequence identity between these two morpholino's.
We have also used a *usp4* ATG (translation) targeting MO (MO1): 5'-CTGCACGATGGCGGAGGAGGCGGACC-3' and MO1 mismatch control: 5'-CTGCACGATCCCGGTCGTGGCGGACC-3′. There is 81% sequence identity between these two morpholino's.
We added these sequences in the Supplementary materials and methods (in the "Primers and reagents").

In response to the reviewer comment we have performed rescue experiments. The MO1 or MO2-induced *usp4* morphant phenotypes were (partly) rescued by the ectopic expression of *usp4* mRNA. The results of these rescue experiments are included as a new Figure (Supplementary Figure S8).

5) The epiboly delay shown in Fig. S7 is quite mild. In fact, the images at 7 hpf show very little delay (the top image of a single embryo is not at the correct angle for comparison with the lower embryo). Epiboly delay is a common MO artifact, and is often seen at high MO doses and may not be observed in the control MO. What MO dose is used in these experiments? Is the delay rescued by mRNA injection? Also, It looks like the embryonic shield does not form in the morphants. Is this the case?

Response: We have corrected the "angle" in Figure S7B.
Our morpholino experiments were carefully controlled; we used a control MO with 4 mismatches compared to the *usp4* MO2. Rescue experiments with *usp4* mRNA were performed and validated before we conducted the functional assays (two independent MOs (MO1 and MO2) targeting *usp4* were used for these experiments).
We also observed a weaker epiboly delay at low *usp4* MO dose compared to the control. In our working concentration, we observed clear shield formation as measured by the expression analysis of the organizer marker *chordin* (Figure F). Thus, we could exclude the possibility of a too high MO concentration as a cause for the failure of organizer formation.
The phenotypes shown in Figure S7B were observed at 5 ng *usp4* MO injection. When we injected *usp4* MO at 2 ng concentration, we still observed an epiboly delay, although it was weaker.

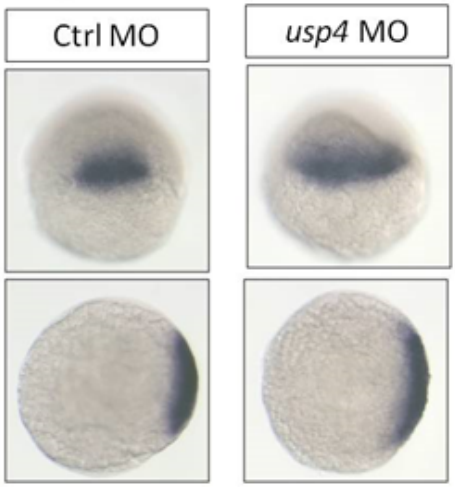


Figure F
Control MO and usp4 MO morphant zebrafish embryos are analysed for *chordin* expression.Top layer: lateral view from organizer; botton layer: animal pole view.

6) How does USP4 effect expression of direct targets of the BMP and Nodal pathways. Expression of Bmp2 in Fig. 1J is good, since BMP2 autoregulates, but another direct target would be helpful (such as Tbox6). Expression of squint and cyclops would also be useful in this context. Does USP4 overexpression lead to an upregulation of these target genes, as predicted?

Response: In our manuscript we showed that *bmp4* mRNA and *bmp7* mRNA induce expression of BMP target genes *bmp2* and *eve1*in zebrafish embryos, and that this ectopic effect is potently inhibited by *usp4* depletion. *Usp4* depletion only weakly inhibits the basal expression of these target genes. We have extended our analysis of the effect of *usp4* misexpression by examining the expression of the BMP target genes *vox* and *vent*. Similar as for the other BMP target genes, we found that *usp4* depletion attenuated the potentiating effect of *bmp4* mRNA and *bmp7* mRNA-induced expression of *vent* and *vox* target genes (Figure G).

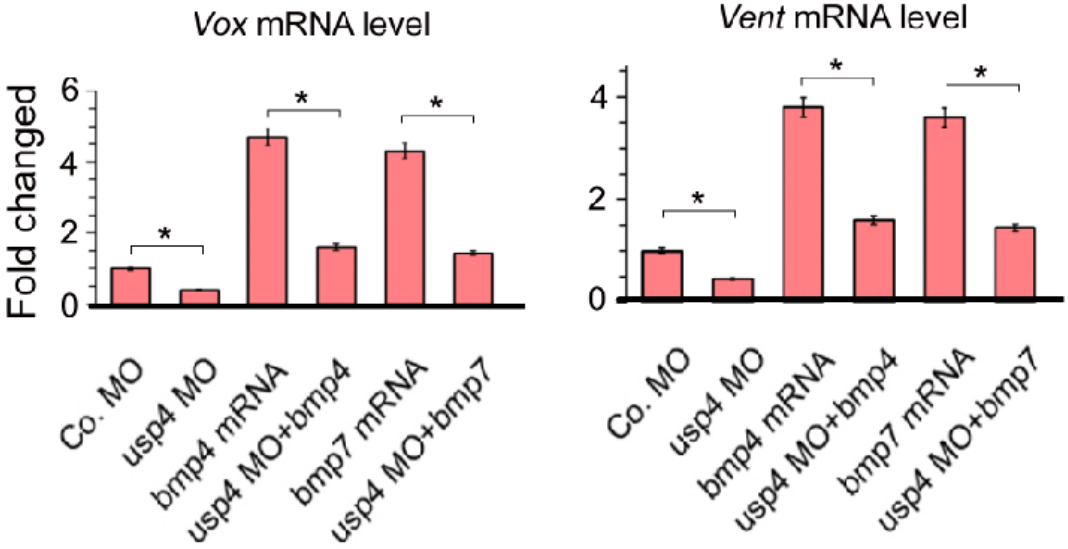


Figure G
Effect of *usp4* depletion on *bmp4* mRNA and *bmp7* mRNA -induced *vox* and *vent* gene expression in zebrafish embryos. The relative expression levels of zebrafish *vox* and *vent* were monitored by real-time PCR (qPCR). Quantified mRNA levels were normalized to *β-actin* and presented relative to control embryos. *P<0.05 indicated the significance levels. Single-cell embryos were injected with control morpholino (MO), *usp4* MO, *bmp4* mRNA or *bmp7* mRNA or their combination (as indicated).

Previously, we have reported that the ectopic expression of *usp4* mRNA in zebrafish strongly promotes the expression of Nodal target genes *goosecoid* (*gsc*) and *notail* (*ntl*) (Zhang et al., 2012). Upon request by the reviewer, we examined the effect of ectopic *usp4* mRNA on BMP target gene expression. We found that the injection of *usp4* mRNA stimulated the expression of BMP target genes *Vox* and *Vent* in zebrafish embryos (new supplementary Figure S2). Thus, *usp4* mRNA potentiates BMP and nodal signaling in zebrafish embryos. This is discussed in the revised version of the paper.

7) There are so many panels in each Figure that the data can be difficult for the reader to see. This problem will be exacerbated on the published page. The zebrafish embryo images shown in Figs. 1I, J, and Fig 7A are especially tiny. I understand the desire to show that the embryos are not cherry picked, but a single representative image should be selected for each stain (WT and Control) and shown in each panel. Leave the multiple embryo panels for the supplement, if necessary.

Response: A single representative image was selected for each stain and shown in each panel. Multiple embryos were shown in the supplementary Figure.

8) Fig. 5D is very confusing. Which part of the gel is the supernatant fraction and which was the pellet. This experiment needs to be described better in the text, and the figure itself should be clarified.

Response: In response to reviewer, we modified both the figure and the text.

9) Does the USP4-CS mutant act like a dominant negative molecule?

Response: We could not observe obvious dominant negative effect of USP4-CS.

Minor Points:

1) Fig. 6D and Fig. 6E show smaller colony sizes in USP4 knockdown mES cells than in controls. But on page 15, the authors claim that this is due to reduced proliferation. There is no evidence to support this conclusion.

Response: Activin and BMP signals suppress differentiation and sustain embryonic stem cell self-renewal; a citation was added and we have modified our description in the first paragraph, Page 14.

2) p8. 'a great portion of Smad4 might be monoubiquinated'. This is a vague statement. Can this be quantified?

Response: We have rephrased the statement that a great portion of SMAD4 might be monoubiquitinated**.**

3) p14. 'depletion led to a much lower activin signal than in wild type mECs... (Fig. 6B)" Again, can this vague statement be quantified?

Response: We quantified the western blotting results by Image J for both Fig 6B and 6C.

There are several typos throughout the manuscript, some of which are confusing to the reader. I list below some of the typos I marked:
p3. "removal from TGF-β family components" should be "removal OF TGF-β"
p7. 'unpon' should be 'upon'
p8. 'monoubiquinated SMAD4 avidly interacts with SMAD4" should read 'monoubiquinated SMAD4 avidly interacts with USP4'

Response: We apologize for those typing mistakes, and corrected them in the revised version of our manuscript.

Referee #2:

This group previously published that USP4 functions to deubiquitinate the TGFbeta type I receptor and thus acts to promote TGFbeta signaling.
In contrast, in this study it is reported that USP4 does not modulate activin/BMP type I receptors rather that USP4 removes mono-ubiquitin on Smad4 and thereby promotes activin/BMP signaling. They go on to show that Smurf2 (which is known to mediate ubiquitination and degradation of several TGFbeta family components), but not Smurf1 or TRIM33, is responsible for Smad4 mono-ubiquitination. A model is presented in which USP4 de-monoubiquitinates Smad4, which promotes Smad complex binding to DNA elements and that this is followed by recruitment of Smurf2 that then monoubiquitnates Smad4 to terminate transcription. Actually, this model is remarkably similar to that proposed by Dupont et al except that the DUB and E3 ligases reported as being responsible in the Dupont study were USP9X and TRIM33, respectively. There is some controversy in the literature as to the precise role of TRIM33 in TGFbeta signaling and this manuscript provides further evidence that our understanding of TRIM33 function in the pathway may require further consideration.

Response: We agree with the reviewer. We clarify previous controversy in the field. Regarding the role of TRIM33/Ectodermin we have included additional discussion and provide an explanation why in our analysis we may have missed in our experimental set up to detect the monoubiquitination of SMAD4 induced by TRIM33/Ectodermin (see also our response to reviewer 1). We could not find

an effect of USP9x on SMAD4 mono-ubiquitination. See also our comment to reviewer1 and new Supplementary Figure S1C and Figure A.

The data presented in this paper in cultured cell lines and mESC supports the conclusion that USP4 enhances activin/BMP signalling. Biochemical data is also presented that is consistent with the idea that USP4 is capable of removing monoubiquitin from Smad4 (with the caveat that additional controls should be included as described below). However, the study would have been enhanced with a more direct demonstration that this is the mechanism whereby USP4 acts to regulate activin/BMP signaling, perhaps through some kind of rescue with a mutant variant of Smad4. Without this, a more cautious title and abstract would be more appropriate

Response: We agree with the reviewer, and have modified and weakened the title.

Finally, there are some results presented which add little to the overall study and rather serve as a distraction, such as data showing binding of other USPs to USP4 and the role of AKT-mediated phosphorylation of USP4 in mESCs. In this reviewer's opinion, these should be removed.

Response: Many thanks for the suggestion. These results have been removed. As a result our manuscript has obtained more focus.

Specific comments:

Fig. 1B. Cell line should be mentioned in the text here rather than later in panel E.

Response: The cell line has been mentioned and also been included in the figure.

Fig. 1C. Knockdown efficiency should be shown.

Response: The shRNA effects could be observed in Fig. 1E. In response to reviewer comment, we demonstrate the knockdown efficiency by QPCR analysis in the new Supplementary Figure S1C**.**

Fig. 1H-K. The authors neglected to indicate in the text that these panels refer to zebrafish embryos. The USP4 knockdown efficiency needs to be included.

Response: We have emphasized that those results are from zebrafish by adding a sentence before we describe the results of Fig. 1H-K: “We then further examined USP4 effects in zebrafish embryos.” USP4 knockdown efficiency by *usp4* targeting morpholino has been validated in our previous publications (Zhou *et al*., 2012; Zhang *et al*., 2012). We mention this in the Supplementary Information (Page 6, the third paragraph).

Page 7. "Indeed, unlike its activity towards TGF-β type I receptor (Zhang et al, 2012b), USP4 did not act as a DUB for activin/BMP type I receptors (data not shown)." Can the authors provide any explanation for this result?

Response: The intracellular domains of the seven type I receptors for TGF-b family members are related with each having their unique intracellular amino acid sequence. The binding partners are overlapping but also distinct as is clear from STRING interaction network in Genecard (http://www.genecards.org/) and also from our own unpublished interactome analysis of each of the type I receptors in the absence or presence of ligand. It is thus possible that USP4 directly interacts with TGF-b type I receptor but not with activin and BMP type I receptors.

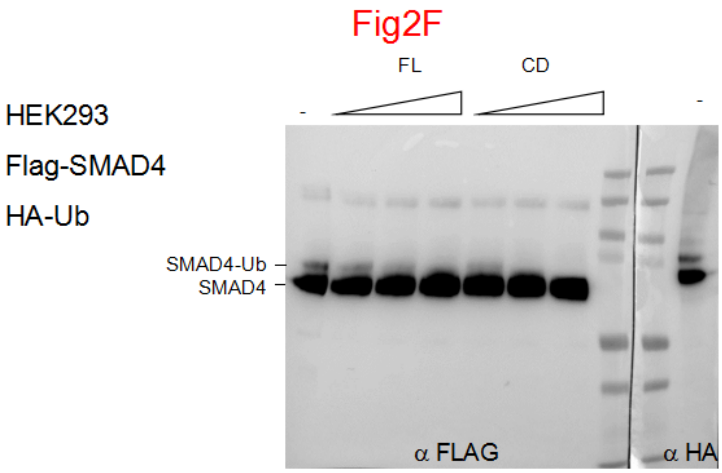
Fig. 2F-J. There is a vertical line marked in all the gel blots that separate the control from the treated samples. Although it is not indicated in the legend, this type of line is typically used to indicate that the blot was spliced. It is extremely concerning that this key control (ie untreated sample) has been spliced in all experiments showing that USP4 can de-ubiquitinate Smad4.

Response: We apologize for the confusion about the vertical lines. Except for one gel in which part of the same gel was blotted with anti-HA and another part was blotted with anti-Flag (old version of Figure 2G, now deleted) and for the gel that was cut in old Supplementary Figure 3B, now new Figure 2G, all the other gels with vertical lines were not part of the original figures (no splicing of

blots). These latter lines have now been removed. See below a detailed description on how the figures were composed from the original findings. We show all the original gels below:

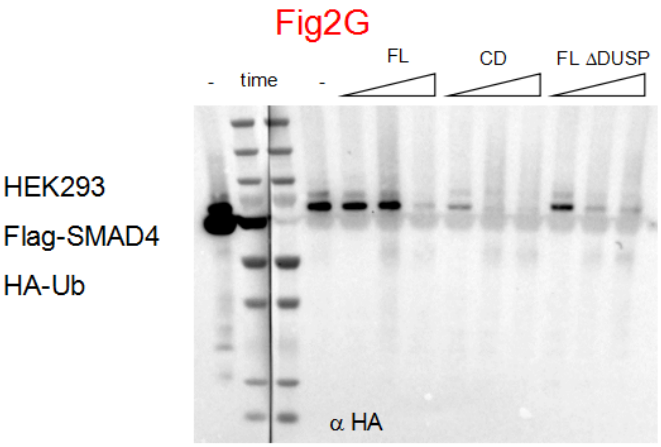
This is original data of Figure 2F:

- Purified monoUb SMAD4

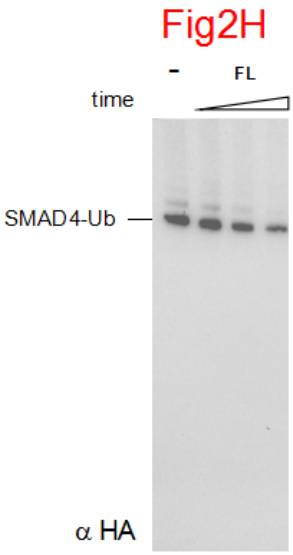


The left 4 lanes were shown as Fig. 2F (immunoblotting with Flag). As shown by the anti-HA blotting, the major ubiquitin-derived modification of SMAD4 is monoubiquitination (Please compare the location of protein bands with the protein markers).

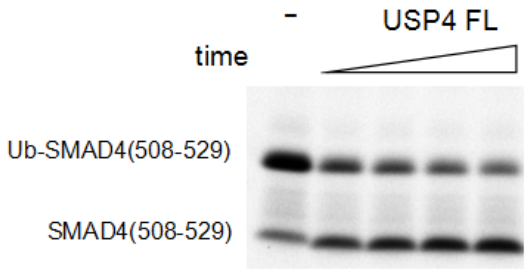
The Figure below is related to old version Figure 2G (now deleted). The left 7 lanes were shown as Figure 2G. In the new version, we have replaced this picture with new data.



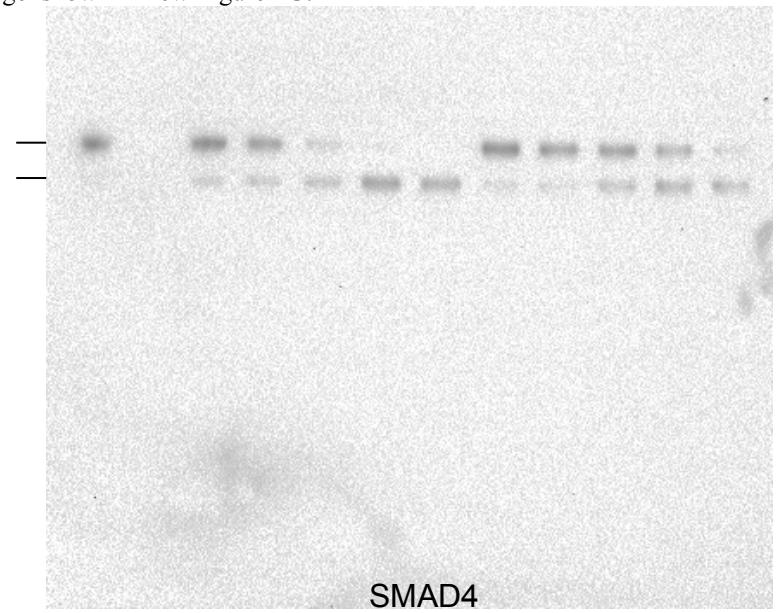
This is original immunoblot of Figure 2H:



This is original gel shown in Figure 2J (signals are measured by fluorescence):



This is original gel shown in new Figure 2G:



The first lane on the left and the last 5 lanes on the right of the gel were combined to generate the Figure as shown in the new Figure 2G.

Fig. 2I. The paper centers on the mono-ubiquitination of Smad4. Thus, a better indication that the HA band is indeed mono-ubiquitinated Smad4 would be useful (ie reblot the HA blot with Flag or

include position of markers and show both blots side by side to indicate relative migration. Also, what is the evidence that this is mono-ubiquitin as opposed to say 2 ubiquitins on Smad4?

Response: Please see the side by side blotting from the original data of Figure 2F (in response to our answer regarding the previous question by this reviewer). We also added new *in vitro* data as Supplementary Figure S3B showing the USP4 mediated the release of free Flag-SMAD4 from purified mono-ubiquitinated Flag-SMAD4. As shown in the Flag immunoblot below (Figure H), in which we analysed purified mono-ubiquitinated Flag-SMAD4 (lane 1), alongside with 293T cells ectopically expressed Flag-SMAD4 (lane 2) and also Flag-SMAD4-Ub fusion protein (lane 3). Mono-ubiquitinated Flag-SMAD4 runs almost at the same size as SMAD4-1xUb fused protein.

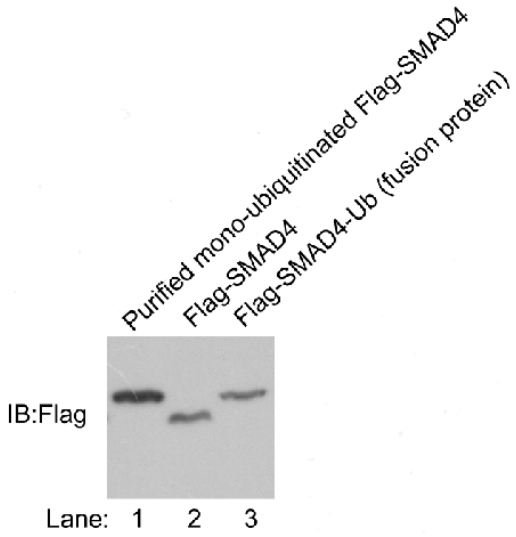


Figure H
Immunoblotting of the purified mono-ubiquitinated Flag-SMAD4 together with free Flag-SMAD4 or Flag-SMAD4 with C-terminal fused with a Ub protein (Flag-SMAD4-Ub).

Fig. 3C-G. To be sure it is ubiquitinated Smads that are being visualized by HA blotting and not simply that the antibody is recognizing total Smad4, a control of cells transfected with SMAD4 but not HA-ubiquitin should be included.
Response: The HA antibody we use does not recognize total SMAD4. For example, in the third lane of Fig. 3B and Fig. 3E: although equal amount of SMAD4 was precipitated (see IP:Flag-IB:Flag), HA-ubiquitin-modified SMAD4 was only detected in the second lane and the fourth lane, but not in the third lane. This indicates that our HA antibody does not recognize total SMAD4. This is also shown in Fig. 2C: the HA antibody specifically reads the ubiquitination of SMAD4, not any bands with the size of the total SMAD4; please note that the Ub ladder of SMAD4 begins from the position about 8-10KD higher than total SMAD4.

Fig. 3D. The importance of testing the mutant forms of ubiquitin was not clear. Perhaps this might be best as part of the supplementary data.

Response: As shown in Fig. 3B, when we overexpressed Flag-SMAD4, we could observe USP4 mediated inhibition of both mono-ubiquitination and poly-ubiquitinaiton of SMAD4. Therefore, we ectopically expressed K48- and K63- ubiquitin chain, in which we clearly showed that USP4 could not inhibit SMAD4 K48- or K63- chain conjugation, but specifically removed SMAD4 mono-ubiquitination. We have adjusted the text accordingly.

The data regarding USP4 stabilization by interacting with USP11/15 adds little to the paper and could be removed.

Response: In response to reviewers comment we have removed this data. We agree with the reviewer that by removing the USP4 interactome results as determined by proteomic/mass spectrometric analysis, the manuscript has gained more focus.

Fig. 4A-B. Do the authors have any pull-down experiments that include a negative control to ensure non-ubiquitinated Smad4 is not binding non-specifically?

Response: We have repeated the experiment and included a negative control; see our new Fig. 4A-B.

Fig. 4A. The legend is not written correctly based on what is in the text. "HEK293T cells transfected with SMAD2 along with SMAD4 or SMAD4-1xUb".

Response: The figure legend was corrected.

Fig. 4D. The text, legends and panel labels are not sufficient to discern what is being shown. What cells were used? Is this endogenous proteins or transfected cells? If transfected, then the ns control is not useful without showing totals.

Response: This result was obtained using non-transfected HEK293T cells. Total input are shown in the left four lanes.

Fig. 6D and E. The requirements of mES cells for activin and/or BMP are rather complex, so the authors need to support their statement (based on literature if available) on how smaller colony sizes and slower proliferation for USP4 depleted cells represents evidence of a defective activin/BMP response.

Response: We revised the description of this part (see also our comments on this issue in response to reviewer 1). Instead of referring to the proliferation speed, we refer to cell numbers (the parameter that was measured).

Page 15 and associated panels relating to AKT. The examination of the putative role of AKT-mediated phosphorylation of USP4 on Smad monoubiquitination and activin/BMP signalling is a side issue and could more appropriately form the basis of a more detailed follow-up study.

Response: As suggested by this reviewer we have removed this part in the revised version. We will publish the regulation of USP4 by its interaction partners (USP11 and USP15) and by its post-translational modification (by AKT phosphorylation) as a separate publication. We agree with the reviewer that our paper has gained focus by removal of these parts.

Other comments:
Al-Salihi et al, Agricola et al and many other references are missing the year.

Response: We have corrected these mistakes

Top of page 4, citation list has an extra set of brackets. Same issue occurs elsewhere.

Response: We have corrected these mistakes.

In the introduction, the paragraph describing the role of c-Ski in Smad complexes and transcription was particularly unclear, ie two particularly problematic sentences in were: "C-SKI inactivates proper R-SMAD-SMAD4 complex formation" and "Inactive SMAD binding to promoters of target genes is promoted by c-SKI"

Response: We have modified the text and clarified this part.

What is the origin of the ARE reporter used in the screen? Presumably, this is not the similarly named Foxh1-dependent reporter (as Foxh1 is not expressed in HEK293T cells).

Response: We co-transfect a FAST2 expression plasmid with the ARE transcriptional reporter to enable the measurement of the activin-induced SMAD2-mediated transcriptional response. SMAD2 does not bind with to DNA directly, but can do indirectly via its DNA binding interaction partner FAST2. We have made this clear in the method “Transcription Reporter Assay” (Page 23). We have

also included a reference for this transcriptional reporter.

SMAD typo (SMDA) on 3rd last line of page 5, Fig. 4C and in other places

Response: We apologized for such mistakes. They have been corrected.

There are missing periods and spaces throughout text.

Response: We apologize for making these mistakes, and have corrected them.

Page 8. A correction is needed of: "SMAD4 avidly interacts with SMAD4"

Response: We apologize for this mistake, and have corrected it.

Methods: A description of how mono-ubiquitinated Smad4 was purified (a key reagent) is missing. The relevant section in the methods erroneously refers to mono-ubiquitinated TBRI purification, but even so no details are provided.

Response: We have made this clear in the method "*In vitro* de-ubiquitination of SMAD4" in Page 23. We apologize for this omission.

Page 13, line 1: 'results' is misspelled

Response: We apologize for this mistake, and have corrected it.

Page 14, line 3 and line 6. The authors refer to 'its' degradation. Since there are several proteins named in the sentences, it would significantly improve clarity if the specific protein that was degraded was actually named (ie c-SKI).

Response: Clarity was provided.

Page 14: The authors need to indicate where the data can be found for this statement: "our observed staining of c-SKI-trapped SMAD4 in the nucleus"

Response: Such description was removed.

Page 14. In this sentence: "USP4 depletion led to a much lower activin signal than in wild type mECs, as shown by a reduction in SMAD2-SMAD4 complex formation" the phrase " a much lower activin signal" is unclear/vague and should be removed.

Response: We have modified the sentence according to the reviewer suggestion.

Figure 7. The presentation of the results of the zebrafish data would be enhanced if the authors would clarify how the observed developmental effects upon loss of USP4 relate to expected changes in activin and/or BMP signalling.

Response: We have included more description linking USP4 misexpression with change in activin and BMP signaling.

For many experiments, the authors neglected to indicate the number of times the experiments were reproduced.

Response: All experiments have been repeated at least three times and representative experiments are shown, this has been made clear in experimental procedures.

Referee #3:

In this paper the authors show that the deubiquitin enzyme USP4 interacts with the co-Smad, Smad4, and catalysis its deubiquitination. Monoubiquitination of Smad4 is found to be induced by

ligand-stimulated R-Smad-Smad4-Smurf2 complex. My major concern about this manuscript, aside from the fact that it is very poorly written, is the novelty. Monoubiquitnation of Smad4 has been reported to be a way of inactivating this co-Smad following complex formation with R-Smads. DUBs have also been reported to stimulate TGF-beta/BMP signaling by allowing R-Smads and Smad4 to recycle back into signaling events. The link between TGF-beta signaling and USP4 was also already known as previous work demonstrated that USP4 acts as a de-ubiqutinase for the type I TGF-beta receptor. As such, and considering that the manuscript is close to a draft, I would not recommend its publication in a high quality journal like EMBO J. The experimental work appears to be well done but it is presented in such a poor way that no coherent story can be derived from the manuscript.

Response: We have rewritten our paper to make the story more coherent.

When re-submitting, the authors would do themselves a favor by proof-reading their manuscript before sending it for publication. The peer-review process is here to ensure the scientific quality of the work and not to correct numerous writing mistakes. Examples of such mistakes can be found below:
- The introduction is poorly written in a telegraphic style. References are wrongly called (multiple et al without the date of publication).

Response: We have corrected the text for typographical errors. We have now properly annotated all the references. We apologize for the mistakes.

- Page 8 "monoubiquitinated SMAD4 avidly interacts with SMAD4").

Response: We apologize for this mistake. This has been corrected.

References:

Agricola E, Randall RA, Gaarenstroom T, Dupont S, Hill CS. (2011) Recruitment of TIF1γ to chromatin via its PHD finger-bromodomain activates its ubiquitin ligase and transcriptional repressor activities. *Mol Cell* 43: 85-96.
Dupont S, Zacchigna L, Cordenonsi M, Soligo S, Adorno M, Rugge M, Piccolo S. (2005) Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell.* 121: 87-99.

Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, Martello G, Stinchfield MJ, Soligo S, Morsut L, Inui M, Moro S, Modena N, Argenton F, Newfeld SJ, Piccolo S. (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFb signaling, controls Smad4 monoubiquitination.*Cell* 136: 123-35

Herhaus L, Al-Salihi MA, Dingwell KS, Cummins TD, Wasmus L, Vogt J, Ewan R, Bruce D, Macartney T, Weidlich S, Smith JC, Sapkota GP (2014) USP15 targets ALK3/BMPR1A for deubiquitylation to enhance bone morphogenetic protein signalling. *Open Biol* 4: 140065

Inui M, Manfrin A, Mamidi A, Martello G, Morsut L, Soligo S, Enzo E, Moro S, Polo S, Dupont S, Cordenonsi M, Piccolo S (2011) USP15 is a deubiquitylating enzyme for receptor-activated SMADs. *Nat Cell Biol* 13: 1368-1375

Kishimoto Y, Lee KH, Zon L, Hammerschmidt M, Schulte-Merker S (1997) The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* 124: 4457-4466

Tse WK, Eisenhaber B, Ho SH, Ng Q, Eisenhaber F, Jiang YJ. (2009) Genome-wide loss-of-function analysis of deubiquitylating enzymes for zebrafish development. *BMC Genomics* 10: 637.

Tse WK, Jiang YJ, Wong CK (2013) Zebrafish transforming growth factor-b-stimulated clone 22 domain 3 (TSC22D3) plays critical roles in Bmp-dependent dorsoventral patterning via two deubiquitylating enzymes Usp15 and Otud4. *Biochim Biophys Acta* 1830(: 4584-4593

Zhang L, Zhou F, Drabsch Y, Gao R, Snaar-Jagalska BE, Mickanin C, Huang H, Sheppard KA, Porter JA, Lu CX, ten Dijke P (2012) USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-b type I receptor. *Nat Cell Biol* 14: 717-726

Zhou F, Zhang X, van Dam H, ten Dijke P, Huang H, Zhang L (2012) Ubiquitin-specific protease 4 mitigates Toll-like/interleukin-1 receptor signaling and regulates innate immune activation. *J Biol Chem* 287: 11002-11010

2nd Editorial Decision 10 February 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been reviewed by referee #2. Referee #2 also looked at your response to the comments raised by referee #1 as this referee was not available to look at the revised version.

As you can see below, the referee appreciates the introduced changes and supports publication here. Please go through the text once more and tidy up any remaining grammatical mistakes, but no new experiments are needed.

That should be all and congratulations on a nice study!

REFEREE REPORT

Referee #2:

The authors have addressed all of my concerns regarding the experimental aspects of the study.

However, while the writing has improved from the previous version, there remain many grammatical errors, such as missing or extra use of the word 'the' and there remain several poorly worded sentences that need attention.

2nd Revision - authors' response 15 March 2017

Referee #2:

The authors have addressed all of my concerns regarding the experimental aspects of the study.

However, while the writing has improved from the previous version, there remain many grammatical errors, such as missing or extra use of the word 'the' and there remain several poorly worded sentences that need attention.
Response: The grammar and language of our manuscript was carefully checked by native English speaker and molecular cell biologist.

Response to Editorial comments:

- We have replaced 5 supplementary Figures with 5 Expanded View figures. Supplementary Figure S1 has become Figure EV1; Supplementary Figure S2 has become Figure EV2; Supplementary Figure S6 has become Figure EV3; Supplementary Figure S4 has become Figure EV4; Supplementary Figure S7 has become Figure EV5.

- We have indicated the number of replicates in the Figure legends.

- We have clarified the black lines in the figures.

- Call out to Figure S8 (Now Appendix Figure S3) is included in the main text in the Materials and Methods section.

- We have included source data on the electrophoretic gels and blots. We have also included the molecular weight markers.

- We included a synopsis of the paper (5 bullet points) and summary figure for the synopsis.

- We checked the title and abstract, and included author checklist.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Peter ten Dijke
EMBO J.
EMBOJ-2016-95372R

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).

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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?	Sample size was determined from previous and pilot experiments. They were used to statistically justify the sample size used in the manuscript.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen on the minum number of animals needed to obtain statistical power.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	If zebrafish died after (mis)injection, they were excluded from the analysis. These criteria were predetermined.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Zebrafish experiments were performed in blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Zebrafish experiments were performed in blinded manner.
5. For every figure, are statistical tests justified as appropriate?	The statistical analysis were performed using a two-tailed, unpaired t-test. P<0.05 was considered statistically significant. The details are provided in the supplemental Experimental procedures.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes.
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 names, catalog numbers, and dillution (for different applications) of all the antibodies used in the study are provided in Materials and Methods and Supplementary information.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of the MEFs is described in materials and methods section. The cell lines were obtained from ATCC. The human cell lines were profiled by STR. All cell lines are regularly 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.	Information on animals are decribed in Matials and Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Under European law the experimentation with zebrafish embyos (up to 5 dpf) is not considered an animal experiment and accordingly did not require the approval by the local animal welfare committee. No experiments were performed on older than 3 dpf and adult zebrafish.
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 have complied with any relevant regulation.

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 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	NA
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. 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: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
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 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

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.	NA