EMBO reports

Wip1 Regulates Smad4 Phosphorylation and Inhibits TGF-β Signaling

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

1st Editorial Decision 15 July 2019

Thank you for the transfer of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, I will not further detail them here. Nevertheless, as EMBO reports emphasises novel functional insight with strong physiological relevance over detailed mechanistic insight, we do not think that point 1 of referee #1 needs to be addressed (of course we welcome additional data in that direction, if you already have those). However, we think that the patho-physiological relevance of the findings indeed needs to be strengthened.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your 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 so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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Moreover, I have these editorial requests:

5) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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7) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors identified Wip1 phosphatase that targets dephosphorylation of Smad4 Thr277. This is an extension of previous work by others. The phosphorylation of Thr277 on Smad4 and its associated effects were published before (see refs in submitted manuscript). The paper is of (potential) general interest if the authors can consolidate their findings, provide more mechanistic insight and demonstrate more conclusive patho-physiological significance of their findings.

Specific major comments:
1. The endogenous SMAD4 Thr277 phosphorylation status using Thr277 phosphorylation (and total SMAD4) specific antibodies upon WIP1 (mis)expression needs to be investigated during development and cell based assays.
2. Why does SMAD4, but not R-SMADs, interact with Wip1 in response to ligand. Can WIP1 compete with R-SMADs for SMAD4 interaction?
3. Which pathological or physiological cues regulate WIP1 expression/activity and thereby affect SMAD4 Thr277 phosphorylation and TGF-beta/BMP signaling? WIP1 is a p53 induced gene and regulator of p53 and perhaps this could be explored.
4. WIP1 expression is manipulated in Xenopus through mRNA or morpholino injection. Ectopic WIP1 RNA/protein expression, or lack thereof, should be shown.
5. WIP1 is suggested to prolong SMAD4 half-life (Fig. 5). The studies need to be extended with pulse chase stability experiments (using cycloheximide). Effect of WIP1 on SMAD4 (phosphorylation and) ubiquitylation status needs to be examined.

Minor comments:

1. The species for expression plasmids used in Xenopus/mammalian cells, such as SMAD4, ALK4, SMAD2. etc. is not clear. Xenopus studies should preferentially use Xenopus expression constructs and human cell studies should use in human expression constructs. Is WIP1/SMAD4 (and its phosphorylation site) highly conserved among species?
2. Figure 2C, K. Does Wip1 knock down affect reporter activity (in absence of ligand stimulation)?
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4. Can dynamic localized endogenous interaction between WIP1 and SMAD4 be shown during development (e.g. using proximity ligation assays)?
5. Can dynamic endogenous interaction between WIP1 and SMAD4 be shown during development?
6. Include molecular weight makers in all the Western blot results.
7. There are a number of inconsistencies in the Western blots. Why are there two bands in P-SMAD4 blot in Fig. 5A, and two bands for pSMAD4 in 5C. Why are there two bands for SMAD4 in Fig. S5B (TCL), and in some other blots SMAD4 runs as one band (e.g. Fig. S5A); why does WIP1 run as two bands in Fig. 2L, S4, and runs as one band in Fig. S5A.

8. Figure S6A, B. Include expression controls for WIP1.

Referee #2:

This manuscript by Park et al demonstrates that the serine/threonine phosphatase WIP1 (PPM1D) influences the TGF-beta/BMP signaling pathways in regulating mesoderm formation and neural induction in early stage Xenopus embryos. They show specifically that WIP1 appears to dephosphorylate T277 on SMAD4, a key signal transducer of TGF-beta/BMP signaling. This dephosphorylation of SMAD4 by WIP1 has multiple effects, but is primarily inhibitory of the usual functions of TGF-beta signaling in development and proliferation.

This paper represents a major new finding in the WIP1 field, as most WIP1 previous targets have been DNA damage response proteins. The technical quality and quantity of the experiments are outstanding. The paper is also very well written. This reviewer is supportive of publication in EMBO Reports.

There are only two issues, one moderate and one minor:

Moderate: The authors' evidence that WIP1 dephosphorylates SMAD4 at T277 is strong, but it is not definitively established that the dephosphorylation is direct rather than indirect (perhaps through another phosphatase) since essentially all the experiments are done in cells or embryos. An in vitro biochemical experiment with purified WIP1 and SMAD4 would be more convincing of a direct relationship. Or perhaps more of the experiments in Fig. 4A/B or 5A/B could be done with the phosphatase-dead version of WIP1 as well as WT WIP1.

Minor point: The authors state in the discussion that: "Smad4 phosphorylation at Thr277 is necessary for mesoderm formation and epidermal differentiation." Given that WIP1 null mice are viable and have only modest defects, it seems that "necessary" is an overstatement. Is it possible that absence of WIP1 in Xenopus is a true embryonic lethal as suggested in Fig. 3? Or are other phosphatases capable of substituting for WIP1?

Referee #3:

Summary: Park et al. report a novel molecular mechanism for regulating Smad4, a central protein in diseases such as cancer and fibrosis. Smad4 is particularly important as it serves as the branching point for BMP and TGF-β signaling pathways, thus, understanding the regulation of Smad4 has broad significance to fields studying either pathway. Smad4 activity is regulated by a critical phosphorylation site, however, the phosphatase enzyme responsible for removing this modification has eluded previous studies. Park et al. report for the first time that Wip1 operates as a phosphatase to remove phosphorylation of Smad4 and regulate downstream signaling. Authors show the critical importance of this dephosphorylation in cultured mammalian cells and in vivo using Xenopus embryos. The strong data using both cultured cells and in vivo models for biochemical and functional assays make this report suitable for EMBO journal.

1. Does this manuscript report a single key finding? YES
This manuscript reports a single new key finding of Wip1 as a phosphatase that regulates Smad4 signaling activity.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES

3. Is it of general interest to the molecular biology community? YES

This report is of interest to the molecular biology community by elucidating a regulatory mechanism for tuning TGFb and BMP signaling pathways through Smad4 activity via post-translational modifications.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)? YES

1. What are the major claims and how significant are they?
Major claims include that Wip1 reduces mesoderm formation for neural induction via BMP and TGF-β inhibition in Xenopus embryos through Smad4. In cultured cell lines, Wip1 regulation og Smad4 reduces TGF-β cytostasis and antimitogenic activity. Further, Wip1 regulates Smad4 nuclear localization and increases Smad4 stability by reducing GSK3 activity, which leads to subsequent degradation. Collectively, Wip1 operates as a regulator of TGF-β signal duration through Smad4.

2. Are the claims novel and convincing?
Previous reports have only identified phosphatases for other Smad proteins. Thus, this work with Wip1 removing Smad4 phosphorylation is novel. The use of dominant negative Wip1 enzyme constructs and Smad4 point mutations, assessed in cell cultured and embyros, provide convincing evidence for this new mechanism.

3. Are the claims appropriately discussed in the context of earlier literature?
The data in this manuscript fit well with previous literature on Smad4 signaling. The manuscript could be strengthened by including a discussion of additional types of post-translational modifications in regulating the duration of growth factor signaling such as arginine methylation of Smad4, Smad 6, and Smad7.

4. Who will be interested and why?
This report will be of interest to fields interested in cancer cell signaling, development, and post-translational modification-regulated protein regulation. Understanding the dynamics of Smad4 activity could provide novel targets for designing pharmaceutical intervention in diseases related to Smad4 protein disruption. Further, Smad4 represents a great molecular example of how the complex interplay between post-translational modifications on a single protein are translated into physiological contexts during tissue morphogenesis and proliferation.

5. Does the paper stand out in some way from the others in its field?
The paper offers unique molecular insight into this signaling mechanism using acceptable methods of analysis.

6. Are the experimental data of sufficient quality to justify the conclusions?
Yes, in particular, embryo experiments were very convincing and rescue embryos were very effective.

1. How the clarity of the writing might be improved (without necessarily going into specific details of spelling and grammar). The final model is very helpful for understanding the complete picture. Authors could also consider showing a small cartoon depiction of Smad4 post-translational modifications with numbered residues and downstream functions (degradation, localization, activity, et cetera) at an earlier point to increase readability for those who are less familiar with Smad4 modifications. Duan is cited twice on page 1. Typo on page one where "whereas" should simply by "where". The use of "Besides, ..." could be revised to improve ease of reading.

2. How the manuscript might be shortened (including the removal of non¬ essential experimental data to supplementary information). Report is acceptable length.

3. How to do the study justice without overselling the claims. Authors do a nice job of describing how this mechanism fits into bigger physiological contexts such as colorectal cancer. Authors could consider including a brief reference to breast cancer and Wip1 expression as these cell lines were specifically chosen for proliferation assay instead of other multiple lines that are also Smad4 null.

4. How to represent earlier literature more fairly. A discussion or acknowledgment of how post-translational modifications other than phosphorylation that have been reported for Smad4, such as arginine methylation, fit into this new mechanism could enhance how this new regulation fits into previous reports.

5. How to improve the presentation of methodological detail so that the experiments can be reproduced. An explanation of the ERK inhibition pulse in the main text during discussions of Figure 5 a could help readers understand the rationale for this experiment. Further, authors may want to include a brief reference of whether one would expect total Smad4 levels to be decreased, in addition to phospho-Smad4, in Fig. 5A at the time points used here. Finally, one might also expect that phospho-Smad4 in lane 1 of both Fig 5. A and B should be more similar. Levels seem to be much lower in lane 1 of Fig. 5B and there is perhaps a doublet for phsophoSmad4 that is absent in A. A little clarification of conditions used for probing with Smad4 phospho-antibodies could strengthen this portion greatly.

1st Revision - authors' response 31 December 2019

Our response to 1st reviewer's comments

The authors identified Wip1 phosphatase that targets dephosphorylation of Smad4 Thr277. This is an extension of previous work by others. The phosphorylation of Thr277 on Smad4 and its associated effects were published before (see refs in submitted manuscript). The paper is of (potential) general interest if the authors can consolidate their findings, provide more mechanistic insight and demonstrate more conclusive patho-physiological significance of their findings.

Specific major comments:

1. The endogenous SMAD4 Thr277 phosphorylation status using Thr277 phosphorylation (and total SMAD4) specific antibodies upon WIP1 (mis)expression needs to be investigated during development and cell based assays.
We have already shown, using HEK293T cells, that forced expression of Wip1 can reduce the levels of Smad4 phosphorylation at Thr277 in FGF2-pulsed (over a period of 2 hours) as well as unstimulated cells (Fig 6A and EV3A). However, the steady-state levels of Smad4 remained constant throughout the time course of the experiment. As suggested, we have also examined the effects of overexpression of Wip1 on this phosphorylation of Smad4 in *Xenopus* embryonic cells. As shown in Fig EV4A, overexpression of wild-type Wip1 could repress FGF8-induced Smad4 phosphorylation at Thr277 in animal caps. Conversely, a phosphatase-dead Wip1 appears to act as a dominant-negative mutant, enhancing Smad4 phosphorylation to some extent in FGF8-stimulated animal cap cells. During a period of 15 hours in this animal cap assay, the level of total endogenous Smad4 was lowered by FGF8 signal, which could be revered by co-expression of wild-type Wip1. These data suggest the specific effects of Wip1 on the phosphorylation status and steady-state levels of Smad4.

2. Why does SMAD4, but not R-SMADs, interact with Wip1 in response to ligand. Can WIP1 compete with R-SMADs for SMAD4 interaction?
To test whether Wip1 interacts with Smad4 in competition with R-Smads, we investigated the effect of Wip1 overexpression on TGF-β-induced association between Smad4 and Smad2. As shown in Fig 5E, TGF-β treatment promoted the interaction between Smad4 and Smad2, which was precluded by co-expression of Wip1. This result suggests Wip1 competition with Smad2 for its interaction with Smad4. On the other hand, it is plausible that the overexpressed Wip1 can dephosphorylate Smad4 at Thr277, thereby leading to disassembly of the Smad4/Smad2 complex. In support of this possibility, recent evidence has shown that Smad4 phosphorylation at the MAPK site enhances R-Smad/Smad4 complex formation (*Cancer Res.*, 2017, 77(6), 1383-94). In our study, wild-type Wip1 associates with Smad4 more effectively than its phosphatase-dead mutant (Fig EV2E), implying that the phosphatase activity of Wip1 is critical for its more efficient interaction with Smad4. Taking together, we suggest that Wip1 acts on Smad4 in complex with R-Smad in the nucleus, resulting in dissociation of R-Smad from Smad4 to control the duration of the transcriptional activity of the R-Smad/Smad4 complex.

3. Which pathological or physiological cues regulate WIP1 expression/activity and thereby affect SMAD4 Thr277 phosphorylation and TGF-beta/BMP signaling? WIP1 is a p53 induced gene and regulator of p53 and perhaps this could be explored.
It has been shown that the major mechanism by which Wip1 activity is modulated is to control the levels of its expression (*Front Biosci.*, 2013, 17, 1480-98). Basal or stress-induced expression of Wip1 is mediated by p53, CREB or NF-kappaB at the transcriptional level. In the early stages of DNA damage response, Wip1 also undergoes microRNA-mediated post-transcriptional regulation; miR-16 binds to the 3' UTR of Wip1 mRNA and promotes its degradation. At the protein level,

Wip1 is phosphorylated by HIPK2, leading to its proteasomal degradation. At the late stages, Wip1 level is increased by reduction of miR-16 expression and escape from HIPK2-driven its degradation. Generally, these up- and down-regulation of Wip1 expression have been shown to occur in response to genotoxic stress. Recently, Li et al. (*Cell Reports*, 2019, 28, 735-45) have reported that DNA damage stabilizes the TGF-β type II receptor via ATM and c-Cbl, thereby enhancing TGF-β signaling. It has been suggested that this DNA damage-induced up-regulation of TGF-β signaling ensures complete cell cycle arrest, allowing efficient DNA damage repair. Given that TGF-β signals can induce ERK-mediated phosphorylation of Smad4 at Thr277, this study indicates the possibility that DNA damage may lead to the linker phosphorylation of Smad4. DNA damage also induces sequential activation of ATM, CHK2 and p53, thereby promoting transcription of Wip1. The increased levels of Wip1 down-regulate ATM, CHK2 and p53 in a negative feedback loop (*Trends Biochem. Sci.*, 2009, 35(2), 109-14). We speculate that DNA damage-induced Wip1 expression would also down-regulate TGF-β signaling by dephosphorylating Smad4 as part of a negative feedback loop. Taken together, these findings suggest that stress signals such as DNA damage may control Smad4 phosphorylation at Thr277 and TGF-β signaling. However, little is known about the cues that regulate Wip1 expression and activity under normal physiological conditions, including early development. As shown in our study, Wip1 displays dynamic spatiotemporal expression pattern in *Xenopus* early development. In mouse, it is present ubiquitously in all adult and embryonic tissues (*Genomics*, 2000, 64, 298-306). As p53 plays critical roles in control of transcription of TGF-β target genes (*Cell*, 2003, 113, 301-14), we examined, as suggested, whether Wip1 would mediate or inhibit p53 induction of gene expression. As shown in Appendix Fig S4, the gain- and loss-of-Wip1 function had no significant effect on the ectopic expression of mesodermal markers induced by p53 in animal caps. This result indicates that Wip1 control of TGF-β signaling does not involve p53 function. Further investigation is necessary to identify the physiological cues and mechanisms underlying control of Wip1 expression.

4. WIP1 expression is manipulated in Xenopus through mRNA or morpholino injection. Ectopic WIP1 RNA/protein expression, or lack thereof, should be shown.
To address this comment, we rechecked whether the levels of Wip1 RNA or protein were altered by injection of its RNA or morpholino oligo (MO). As shown in Fig 1A-C, higher levels of Wip1 RNA were observed in its wild-type or mutant RNA-injected animal caps than in uninjected control caps. This injection of Wip1 RNAs led to an increase in the levels of its protein (Fig EV4B and C, and Appendix Fig S1B). Conversely, injection of *Wip1* MO caused less production of its exogenous and endogenous proteins (Fig 6D and Appendix Fig S1A). These data support that Wip1 expression could be efficiently manipulated by injection of its RNA or MO in our gain- and loss-of-function analyses.

5. WIP1 is suggested to prolong SMAD4 half-life (Fig. 5). The studies need to be extended with pulse chase stability experiments (using cycloheximide).
As commented, we have performed cycloheximide time course experiments to test whether Wip1 extends the half-life of Smad4. As shown in Figs 6F, and EV3D, Smad4 exhibited a reduction in half-life in the absence of Wip1 in FGF2-stimulated cells, consistent with Fig 6D. These results indicate that Wip1 plays a critical role in prolonging the stability of Smad4.

6. Effect of WIP1 on SMAD4 (phosphorylation and) ubiquitylation status needs to be examined.
The effects of the gain- and loss-of-function of Wip1 on the phosphorylation of Smad4 at Thr277 have already been shown in Fig 6A and B. This phosphorylation of Smad4 has been shown to prime GSK3-mediated phosphorylation of Smad4 at sites close to Thr277, which leads to its polyubiquitination and proteasome-dependent degradation (*Cell Reports*, 2014, 9, 688-700). Since knockdown of Wip1 caused decreased levels of Smad4 as in Fig 6D, highly ubiquitinated forms of Smad4 might be observed in the absence of Wip1. As expected, silencing of Wip1 yielded multiubiquitinated conjugates of wild-type Smad4 but not of Smad4(T277A) mutant (Fig 6E). Thus, these results indicate that Wip1-mediated dephosphorylation of Smad4 at Thr277 is essential for regulation of its ubiquitination status and turnover.

Minor comments:

1. The species for expression plasmids used in Xenopus/mammalian cells, such as SMAD4, ALK4, SMAD2. etc. is not clear. Xenopus studies should preferentially use Xenopus expression constructs and human cell studies should use in human expression constructs. Is WIP1/SMAD4 (and its phosphorylation site) highly conserved among species?
To address this point, we have described in the Materials and Methods the species from which gene sequences originated. While as commented, *Xenopus* and human expression constructs have been mostly used for *Xenopus* embryonic assays and human cell studies, respectively, some highly conserved genes such as human Smad4 have been used for our *Xenopus* studies. Wip1 and Smad4 genes are conserved well. Sequence comparison shows that human Smad4 is 90 and 72% identical at the amino acid level to the *Xenopus* Smad4α and Smad4β, respectively. Furthermore, the

consensus ERK phosphorylation site in the linker region of Smad4 is also conserved between humans and frogs. Human Wip1 shares 50% amino acid identity with *Xenopus* Wip1. Consistent with our data in this study, these sequence similarities suggest the conserved functions of Smad4 and Wip1 among species.

2. Figure 2C, K. Does Wip1 knock down affect reporter activity (in absence of ligand stimulation)?

To answer this question (regarding Fig 2C), we checked whether depletion of Wip1 would affect the activity of the activin-responsive reporter (ARE3-luc) in unstimulated HEK293T cells. FAST-1 cofactor whose expression is critical for activation of the reporter was transfected with siRNAs. As shown in Appendix Fig S3, expression of FAST-1 led to a small increase of the reporter activity, which was not affected by silencing of Wip1. Since FAST-1-induced changes in the levels of the reporter activity appear to be marginal, compared to those in ligand-stimulated cells, this new result has been included separately in Appendix Figs. We also investigated the effects of Wip1 knockdown on the proliferation of HEK293T cells in the absence of TGF-β stimulation. As shown in new Fig 3B in revised version, depletion of Wip1 enhanced significantly TGF-β-induced cytostasis but had no effect on the growth of HEK293T cells without ligand activation. Of note, silencing of Wip1 promoted significantly the migration and invasion of MDA-MB231 breast cancer cells even in the absence of ligand stimulation (Fig 3C-F). This breast cancer cell has low levels of Smad4 and displays attenuated TGF-β responsiveness. Taking together, we speculate that the loss-of-Wip1 function could affect cell growth and/or migratory behaviors, depending on the cell types.

3. Figure S5B. IP-WB, most right lane. What do the two bands represent?

The initial Fig S5B is currently labeled as Fig EV2B. As pointed out, there are two bands for Myc-Smad4 in immunoprecipitates as well as total cell lysates. Of the two bands, the lower band appears to represent the Myc-tagged Smad4 with a predicted molecular weight. However, the upper band seems not to be a non-specific band, given that this band is not observed in the other lanes. We speculate that the upper band may indicate a post-translationally modified form of Smad4. We do not know exactly which modified form this upper band represents. One possible form is a monoubiquitinated Smad4 whose molecular weight is approximately 84 kDa (*Cell*, 2009, 136, 123-135). In this co-immunoprecipitation experiment, Wip1 was overexpressed to detect its interaction with Smad4. As shown in our study, overexpressed Wip1 down-regulates Smad4 phosphorylation at Thr277. Recently, this linker phosphorylation of Smad4 has been shown to promote the ability of USP9x, a deubiquitinating enzyme to bind Smad4 in competition with TIF1γ, a monoubiquitin ligase, enhancing Smad4 deubiquitination (*Cancer Res.*, 2017, 77(6), 1383-94). These results suggest the dynamic control of Smad4 ubiquitination by its phosphorylation status. It is plausible that Smad4 would be much more highly dephosphorylated and then monoubiquitinated in Wip1-overexpressing cells than in control cells. TIF1γ ubiquitinates Smad4 more actively in the presence of TGF-β signals (*Cell*, 2009, 136, 123-135). Our data also show that co-expression of *Xnr1* produced the upper band as well as the lower band. The experiments shown in Fig EV2B were performed using *Xenopus* animal caps, which have normally active BMP signaling. Without Xnr1 expression, this high activity of BMP might contribute to generation of the upper band in Wip1-overexpressing animal caps as shown in total cell lysate. In many of our experiments, immunoprecipitates or total cell lysates were resolved by 8% SDS-PAGE to detect Smad4 or phospho-Smad4. In this case, the proteins were separated in 6% SDS-PAGE. Use of this lower percentage gel might reveal the upper band that was invisible in higher percentage gel. Taking together, we have not mentioned in the text that the upper band represents a post-translationally modified version of Smad4 but have inserted a new bracket symbol to indicate that both the upper and lower bands represent Myc-Smad4.

4. Can dynamic localized endogenous interaction between WIP1 and SMAD4 be shown during development (e.g. using proximity ligation assays)?

As suggested, we performed proximity ligation assays (PLA) to examine the dynamic association between endogenous Wip1 and Smad4. At first, we tried to observe their localized interaction in *Xenopus* animal cap cells (because the reviewer asked if their association can be shown during development) but failed to obtain satisfactory results, probably owing to the features of the embryonic cells, such as relatively larger thickness, and the fact that the kit (Duolink PLA kit) we used is more suitable for experiments with cell lines. Thus, we used HeLa cells instead for these assays. Without TGF-β stimulation, a few spots, which indicate the localized interaction between Wip1 and Smad4, were visible in the nucleus of HeLa cells (Fig 5C and D). Given that Smad4 shuttles continuously between the nucleus and the cytoplasm independently of TGF-β signaling (*Mol. Cell Biol.*, 2000, 20(23), 9041-54), it is possible that Wip1 and Smad4 exhibit their interaction even in unstimulated cells, albeit less than in stimulated cells. Co-immunoprecipitation experiments also showed their association in the absence of ligand activation (Fig 5B and EV2E). Upon treatment with TGF-β1, a highly increased number of spots were detectable in the nucleus of the cells, indicative of ligand-induced more interaction between the two proteins. Together with IPs, the PLA supports the dynamic association of Wip1 and Smad4 at physiological levels of their expression. We hope that this positive result will meet the request of the reviewer to show dynamic interaction during development.

5. Can dynamic endogenous interaction between WIP1 and SMAD4 be shown during development?
Since the question 4 and 5 are the same, please see our response to the above question.

6. Include molecular weight makers in all the Western blot results.
As suggested, molecular weights have been indicated in the Western blot data.

7. There are a number of inconsistencies in the Western blots. Why are there two bands in P-SMAD4 blot in Fig. 5A, and two bands for pSMAD4 in 5C.Why are there two bands for SMAD4 in Fig. S5B (TCL), and in some other blots SMAD4 runs as one band (e.g. Fig. S5A); why does WIP1 run as two bands in Fig. 2L, S4, and runs as one band in Fig. S5A.
Since this point is similar to the minor 3rd comment, please see our response to the above comment for details. As we have described above, Smad4 seems to be modified dynamically depending on the status of its linker phosphorylation; phosphorylation of Smad4 at Thr277 promotes its deubiquitination, whereas Smad4 dephosphorylation at the site leads to its monoubiquitination (*Cancer Res.*, 2017, 77(6), 1383-94). In addition, phosphorylation of Smad4 at the MAPK site acts to prime GSK3-mediated phosphorylation of three threonine residues near the MAPK site (see Fig 6G). This GSK3 phosphorylation causes β-TrCP-mediated polyubiquitination of Smad4 and its subsequent proteasome-dependent degradation. Given that depletion of Wip1 promotes polyubiquitination of Smad4 and its turnover (Fig 6E and F), it is possible that the sequential GSK3 phosphorylation of Smad4 at the Thr residues would be highly enhanced in Wip1-silenced cells. Thus, of the two bands for phospho-Smad4 in Fig 5C (now labeled as Fig 6B), the upper band appears to represent Smad4 dually phosphorylated both at the MAPK and GSK3 sites. In Co siRNA-transfected cells, treatment with FGF2 alone also generated the upper bands whose levels were further enhanced in Wip1-depleted cells. Furthermore, in the experiments shown in Fig 6B, the proteins were resolved by 6% SDS-PAGE, which might make it possible to see clearly the two bands that were not distinguishable in higher percentage gels. For Figs 6A and EV4A, the samples were separated in 8% SDS-PAGE, and these results show the levels of phospho-Smad4 in Wip1-overexpressing cells. These might be the reason why the bands for phospho-Smad4 in Fig 6A and B look different from each other. We have inserted newly a bracket in Fig 6B to indicate that the upper and lower bands all represent phospho-Smad4.
As for Wip1, it seems that while one of the two bands is the actual Wip1, the other represents not a modified form of Wip1 but a non-specific band. The data in Fig 2L and S4 (currently labeled as Fig 2K and L, respectively) were obtained from western blotting with anti-Wip1 antibody, but the western blot result in Fig S4A (now labeled as Fig EV2A) was produced by using anti-Flag antibody. This may be the reason why the bands for Wip1 look different between Figs 2K and L, and EV2A. In addition, a few bands generated by anti-Wip1 antibody in our western blotting appear to be non-specific based on their molecular weights and/or changes in their levels in response to Wip1 depletion. Thus, we have inserted newly arrows to indicate the non-specific bands in Fig 2K and L, and 6F.

8. Figure S6A, B. Include expression controls for WIP1.
Following this comment, we have included new data to show increased expression levels of Wip1, which were caused by injection of wild-type *Wip1* or *Wip1(D277A)* mRNA, compared to those of its endogenous expression in animal caps. Previous Fig S6A and B are currently labeled as Fig EV4B and C, respectively, in revised version.

Our response to 2nd reviewer's comments

This manuscript by Park et al demonstrates that the serine/threonine phosphatase WIP1 (PPM1D) influences the TGF-beta/BMP signaling pathways in regulating mesoderm formation and neural induction in early stage Xenopus embryos. They show specifically that WIP1 appears to dephosphorylate T277 on SMAD4, a key signal transducer of TGF-beta/BMP signaling. This dephosphorylation of SMAD4 by WIP1 has multiple effects, but is primarily inhibitory of the usual functions of TGF-beta signaling in development and proliferation. This paper represents a major new finding in the WIP1 field, as most WIP1 previous targets have been DNA damage response proteins. The technical quality and quantity of the experiments are outstanding. The paper is also very well written. This reviewer is supportive of publication in EMBO Reports. There are only two issues, one moderate and one minor:

Moderate: The authors' evidence that WIP1 dephosphorylates SMAD4 at T277 is strong, but it is not definitively established that the dephosphorylation is direct rather than indirect (perhaps through another phosphatase) since essentially all the

experiments are done in cells or embryos. An in vitro biochemical experiment with purified WIP1 and SMAD4 would be more convincing of a direct relationship.
Following this suggestion, we performed an *in vitro* phosphatase assay to test whether Wip1 could directly dephosphorylate Smad4 at Thr277. As shown in Fig 6C, phosphorylated Smad4, which was immunopurified from the cell lysates, could be efficiently dephosphorylated by *in vitro* incubation with recombinant Wip1 protein in the presence of Mg^{2+}. In contrast, Wip1 failed to dephosphorylate the phospho-Smad4 in the absence of this metal-ion. Thus, these results suggest that phospho-Smad4 at Thr277 is a direct target of Wip1.

Or perhaps more of the experiments in Fig. 4A/B or 5A/B could be done with the phosphatase-dead version of WIP1 as well as WT WIP1.
As an extension of our initial Fig 4A and B, which are currently labeled as Fig 5A and B in revised manuscript, more immunoprecipitation experiments were carried out to examine whether like wild-type Wip1, Wip1(D314A), the phosphatase-inactive mutant of human Wip1, would interact with Smad4. As shown in Fig EV2E, this Wip1 mutant still binds to Smad4, though their association seems to be weaker and inefficient, compared to that of wild-type Wip1 and Smad4. Non-phosphorylatable Smad4 also retains the ability to interact with Wip1, albeit more weakly than wild-type Smad4 (Fig EV2C). The mutations appear to cause structural changes, leading to interference with their effective interaction. Thus, in our view, the phosphatase activity of Wip1 is essential for its effective association with Smad4.
The initial Fig 5A and B, which are labeled as Fig 6A and EV3A, respectively, in current version, demonstrated wt Wip1-mediated down-regulation of Smad4 phosphorylation at Thr277. In contrast, Wip1(D277A), the phosphatase-dead mutant of *Xenopus* Wip1, seems to act in a dominant negative manner to increase the levels of phosphorylated Smad4 to a small extent in FGF8-stimulated embryonic cells (Fig EV4A). As our response to the second suggestion, this new Fig EV4A has been included in revised version instead of the time course experiments shown in the previous Fig 5A and B.

Minor point: The authors state in the discussion that: "Smad4 phosphorylation at Thr277 is necessary for mesoderm formation and epidermal differentiation." Given that WIP1 null mice are viable and have only modest defects, it seems that "necessary" is an overstatement. Is it possible that absence of WIP1 in Xenopus is a true embryonic lethal as suggested in Fig. 3? Or are other phosphatases capable of substituting for WIP1?
As shown in Fig 4, the defective phenotypes of Wip1-depleted embryos can be rescued by co-expression of MO-resistant *Wip1* RNA, supporting the specific effects of *Wip1* MO on *Xenopus* early development. In line with its effects at the molecular levels (Fig 2A and B, and 4B and D), injection of *Wip1* MO produced the phenotypes resembling those of embryos exposed to increased levels of TGF-β signals. Our results suggest that depletion of Wip1 generates these phenotypes by eliciting a prolonged phosphorylation of Smad4 at Thr277 and its enhanced transcriptional activity. Demagny et al. (*Cell Reports*, 2014, 9, 688-700) showed that this linker phosphorylation of Smad4 is required for its maximal transcriptional activity and also primes it for GSK3 phosphorylation, leading to transcriptional inhibition and the E3-ligase β-TrCP-mediated polyubiquitiation and subsequent degradation of Smad4. In the same study, injection of a Smad4 mutant, which has the intact Thr277 but GSK3 sites mutated into phosphorylation-resistant residues, caused a strong expansion of mesoderm and Spemann organizer region in embryos depleted of endogenous Smad4. This result is consistent with the devastating effect of Wip1 knockdown on the embryonic phenotypes.
In *Xenopus* embryogenesis, Wip1 transcripts are more abundant at the blastula and gastrula stages than later stages (Appendix Fig S2). Germ layer specification and body axis formation actively occur during these earlier stages. Given this temporal expression pattern of *Wip1* and its critical roles in germ layer formation, the malformed phenotypes of Wip1 morphants shown in Fig 4 might be due to the long-term effects of its depletion at earlier stages. In contrast, Wip1-null mice are viable and exhibit only postnatal modest defects in reproductive organs, cell cycle control and immune function as mentioned by the referee. While Wip1 transcripts have been shown to be present ubiquitously in all mouse adult and embryonic tissues (*Genomics*, 2000, 64, 298-306), it is not clear whether Wip1 is expressed in the right place at the right time for formation of germ layers and body axis in mouse early embryonic development. Thus, a likely explanation to account for the discrepancy between Wip1-depleted frogs and Wip1-null mice is the possibility of different spatiotemporal expression patterns of Wip1 between two species in early development. However, regardless of whether its expression patterns in two species are similar or not, the importance of Smad4 for mesoderm formation in mice (*Development*, 2004, 131, 3501-12) and of Smad4 linker phosphorylation for its transcriptional activity strongly suggest functional redundancy or compensation by other phosphatases in Wip1-null mice. PPM1A, a close member of the PP2C protein phosphatase family, has been shown to dephosphorylate the C-terminal SSXS motifs of Smad1 and Smad2/3 (*CSH Perspect. Biol.*, 2016, 6, a022087). In addition, the small C-terminal domain phosphatases (SCPs) act on the Smad1 but the Smad2/3 C-terminal sites. Pyruvate dehydrogenase phosphatase (PDP), a mitochondrial protein, dephosphorylates the homolog of Smad1 in *Drosophila*. Furthermore, the SCPs dephosphorylate the linker regions of Smad1 and

Smad2/3. Given these findings, it is possible that another phosphatase might be redundantly involved in dephosphorylation of Smad4 at Thr277 during germ layer formation in mouse early development. In *Xenopus*, this additional phosphatase, which has yet to be identified, may be absent at early stages and not be capable of compensating the loss of Wip1 for normal development. Taken together, it still appears reasonable to state that "Smad4 phosphorylation at Thr277 is necessary for mesoderm formation and epidermal differentiation."

Our response to 3rd reviewer's comments

Summary: Park et al. report a novel molecular mechanism for regulating Smad4, a central protein in diseases such as cancer and fibrosis. Smad4 is particularly important as it serves as the branching point for BMP and TGF-β signaling pathways, thus, understanding the regulation of Smad4 has broad significance to fields studying either pathway. Smad4 activity is regulated by a critical phosphorylation site, however, the phosphatase enzyme responsible for removing this modification has eluded previous studies. Park et al. report for the first time that Wip1 operates as a phosphatase to remove phosphorylation of Smad4 and regulate downstream signaling. Authors show the critical importance of this dephosphorylation in cultured mammalian cells and in vivo using Xenopus embryos. The strong data using both cultured cells and in vivo models for biochemical and functional assays make this report suitable for EMBO journal.

We appreciate the reviewer's positive evaluation of our work.

1. Does this manuscript report a single key finding? YES
This manuscript reports a single new key finding of Wip1 as a phosphatase that regulates Smad4 signaling activity.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES

3. Is it of general interest to the molecular biology community? YES
This report is of interest to the molecular biology community by elucidating a regulatory mechanism for tuning TGFb and BMP signaling pathways through Smad4 activity via post-translational modifications.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer format article (NO)? YES

1. What are the major claims and how significant are they?
Major claims include that Wip1 reduces mesoderm formation for neural induction via BMP and TGF-β inhibition in Xenopus embryos through Smad4. In cultured cell lines, Wip1 regulation of Smad4 reduces TGF-β cytostasis and antimitogenic activity. Further, Wip1 regulates Smad4 nuclear localization and increases Smad4 stability by reducing GSK3 activity, which leads to subsequent degradation. Collectively, Wip1 operates as a regulator of TGF-β signal duration through Smad4.

2. Are the claims novel and convincing?
Previous reports have only identified phosphatases for other Smad proteins. Thus, this work with Wip1 removing Smad4 phosphorylation is novel. The use of dominant negative Wip1 enzyme constructs and Smad4 point mutations, assessed in cell cultured and embryos, provide convincing evidence for this new mechanism.

3. Are the claims appropriately discussed in the context of earlier literature?
The data in this manuscript fit well with previous literature on Smad4 signaling. The manuscript could be strengthened by including a discussion of additional types of post-translational modifications in regulating the duration of growth factor signaling such as arginine methylation of Smad4, Smad 6, and Smad7.
Following this comment, we have included a new introduction and discussion of how methylation of arginine residues in Smad4, Smad6 and Smad7 contributes to control of TGF-β signaling (page 3, line 9 from the top; page 4, line 12 from the top; page 18, line 5 from the top).

4. Who will be interested and why?
This report will be of interest to fields interested in cancer cell signaling, development, and post-translational modification-regulated protein regulation. Understanding the dynamics of Smad4 activity could provide novel targets for designing pharmaceutical intervention in diseases related to Smad4 protein disruption. Further, Smad4 represents a great molecular example of how the complex interplay between post-translational modifications on a single protein are translated into physiological contexts during tissue morphogenesis and proliferation.

5. Does the paper stand out in some way from the others in its field?
The paper offers unique molecular insight into this signaling mechanism using acceptable methods of analysis.

6. Are the experimental data of sufficient quality to justify the conclusions?
Yes, in particular, embryo experiments were very convincing and rescue embryos were very effective.

1. How the clarity of the writing might be improved (without necessarily going into specific details of spelling and grammar). The final model is very helpful for understanding the complete picture. Authors could also consider showing a small cartoon depiction of Smad4 post-translational modifications with numbered residues and downstream functions (degradation, localization, activity, et cetera) at an earlier point to increase readability for those who are less familiar with Smad4 modifications.
Following this comment, we have incorporated a new small diagram showing the post-translational modifications of Smad4 in Fig 6G.

Duan is cited twice on page 1. Typo on page one where "whereas" should simply by "where". The use of "Besides, ..." could be revised to improve ease of reading.
There seems to be a misunderstanding that the same paper is cited twice at the end of a sentence. The two cited papers are different. The typo the reviewer pointed out, “whereas”, seems to be one in the following sentence, “Unlike R-Smads, Smad4 undergoes no C-terminal tail phosphorylation, whereas it is phosphorylated in the linker region”. While we think that the word “whereas” is correct grammatically and in context, it has been replaced by the word “though” to avoid confusion. To improve the readability of the sentence mentioned by the referee, it was divided into two sentences and revised as follows; “Besides, the FGF/MAPK pathway phosphorylates Smads 1, 2 and 3 in the linker region between the MH1 and MH2 domains. This linker phosphorylation prevents nuclear accumulation of R-Smads and Smad-dependent transcriptional responses induced by agonists such as TGF-β and BMP ”.

2. How the manuscript might be shortened (including the removal of non-essential experimental data to supplementary information). Report is acceptable length.

3. How to do the study justice without overselling the claims. Authors do a nice job of describing how this mechanism fits into bigger physiological contexts such as colorectal cancer. Authors could consider including a brief reference to breast cancer and Wip1 expression as these cell lines were specifically chosen for proliferation assay instead of other multiple lines that are also Smad4 null.
Wip1 has been found to be amplified and more recently mutated in a variety of human cancers including breast tumors (*Trends Biochem. Sci.*, 2009, 35(2), 109-14). While MDA-MB231 breast cancer cells retain low levels of Smad4 and display attenuated TGF-β responsiveness, they have been frequently used to study TGF-β signaling pathway. In addition, they increase their motility in response to TGF-β signaling, which is useful for cell migration/invasion assays. That’s why we used this cancer cell line for our study. As commented, we have included a new short description of breast cancer and Wip1 expression in the text (page 8, line 6 from the bottom).

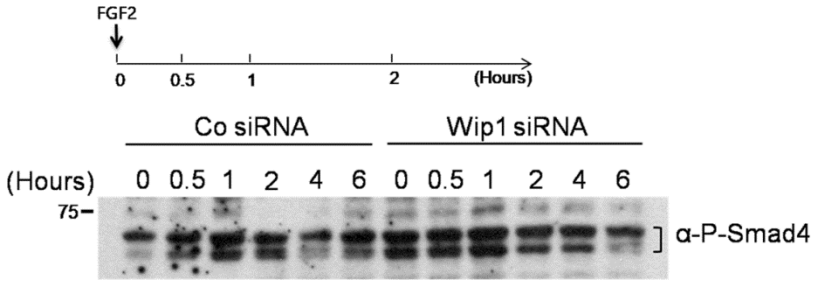
4. How to represent earlier literature more fairly. A discussion or acknowledgment of how post-translational modifications other than phosphorylation that have been reported for Smad4, such as arginine methylation, fit into this new mechanism could enhance how this new regulation fits into previous reports.
As commented, we have included a new introduction and discussion of the contributions that Arg methylation of Smads makes to control of TGF-β signaling, and of the relevance that Wip1 dephosphorylation of Smad4 has to its Arg methylation (page 3, line 9 from the top; page 4, line 12 from the top; page 18, line 5 from the top).

5. How to improve the presentation of methodological detail so that the experiments can be reproduced.

An explanation of the ERK inhibition pulse in the main text during discussions of Figure 5 a could help readers understand the rationale for this experiment.
Right after the pulse of FGF2, cells were treated with U0126, a MEK inhibitor to prevent Smad4 phosphorylation by the residual ERK activity and to analyze the changes in the levels of its phosphorylation induced only for a short period of time. As suggested, this explanation has been included in the main text (page 12, line 2 from the top).

Further, authors may want to include a brief reference of whether one would expect total Smad4 levels to be decreased, in addition to phospho-Smad4, in Fig. 5A at the time points used here.
At first glance, one would speculate that the decreased levels of phospho-Smad4 may be due to Wip1-mediated reduction of total Smad4 but not to its negative effect on Smad4 phosphorylation. However, the steady-state levels of Smad4 remained unchanged during the time-course studied (new Fig 6A). This result indicates the immediate inhibitory effect of Wip1 on the linker phosphorylation of Smad4. This point has been newly described in the text (page 12, line 9 from the top).

Finally, one might also expect that phospho-Smad4 in lane 1 of both Fig 5A and B should be more similar. Levels seem to be much lower in lane 1 of Fig. 5B and there is perhaps a doublet for phsophoSmad4 that is absent in A. A little clarification of conditions used for probing with Smad4 phospho-antibodies could strengthen this portion greatly.
We agree with the point that the levels of phospho-Smad4 in unstimulated control cells should look similar in Fig 5A and C (now labeled as Fig 6A and B). In our analysis, longer exposures of the same blot in Fig 6B resulted in the comparable levels of bands to that shown in Fig 6A as below.



To reveal more clearly the enhancing effects of Wip1 depletion on the levels of phospho-Smad4, we showed a current image from shorter exposures in Fig 6B instead of the saturated bands from longer exposures. As pointed out, there are two bands for phospho-Smad4 in Fig 6B. It is of note that these two bands exhibit similar patterns of changes in their levels throughout the time course of the experiment. A possible reason for the doublet for phospho-Smad4 in Fig 6B is that Smad4 can be modified dynamically depending on the status of its linker phosphorylation. In particular, phosphorylation of Smad4 at Thr277 acts to prime GSK3-mediated phosphorylation of three threonine residues near the MAPK site (see Fig 6G). This GSK3 phosphorylation causes β-TrCP-mediated polyubiquitination of Smad4 and its subsequent proteasome-dependent degradation (*Cell Reports*, 2014, 9, 688-700). Given that depletion of Wip1 promotes polyubiquitination of Smad4 and its turnover (Fig 6E and F), it is possible that the sequential GSK3 phosphorylation of Smad4 at the Thr residues would be highly enhanced in Wip1-silenced cells. Fig 6A and B show the changes in the levels of phospho-Smad4 in Wip1-overexpressing and -depleted cells, respectively. Thus, of the two bands for phospho-Smad4 in Fig 6B, the upper band appears to represent Smad4 dually phosphorylated both at the MAPK and GSK3 sites. In Co siRNA-transfected cells, treatment with FGF2 alone also generated the upper bands whose levels were further enhanced in Wip1-depleted cells. Another point to mention is that for phospho-Smad4 in Fig 6B, the proteins were resolved by 6% SDS-PAGE. However, the samples were separated in 8% SDS-PAGE for Fig 6A. Use of the lower percentage gels might make it possible to see clearly the two bands that were not distinguishable in higher percentage gels. These might be the reason why the bands for phospho-Smad4 in Fig 6A and B look different from each other. We have inserted newly a bracket in Fig 6B to indicate that the upper and lower bands all represent phospho-Smad4.

2nd Editorial Decision 27 January 2020

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all three referees now support the publication of the study in EMBO reports.

Before we can proceed with formal acceptance, I have these final editorial requests:

- I would suggest a shorter title:
'(Phosphatase) Wip1 regulates Smad4 phosphorylation and inhibits TGF-β signalling'
or
'Wip1 dephosphorylates Smad4 and inhibits TGF-β signalling'

- Please provide scale bars for all microscopic images. Most of these currently have no scale bars (e.g. Figs. 1F-Q, 2E-J, 3C, 3E, 4A-I, 5C, 7A-J, EV1A-E and S2B-D). These are presently hardly visible. Please refrain from writing the size on or near the scale bars in the image. Please add the size information only to the respective figure legend.

- There seems to be no call out for Figure 5D. Please check.

- Please indicate in all the figure legends if the replicate experiments mentioned are technical or biological. This is not clear presently; as it is only mentioned they were 'independent'.

- Per journal policy, we do not allow 'data not shown' (see page 17 and 21 of your manuscript). All data referred to in the paper should be displayed in the main or Expanded View figures, or in the Appendix. Thus, please add these data (or change the text accordingly, if these data are not important). See:
http://www.embopress.org/page/journal/14693178/authorguide#unpublisheddata

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have addressed (nearly) all comments that were raised by the reviewers. The paper is now acceptable for publication.

Referee #2:

This reviewer had only one moderate and one minor issue with the original submitted manuscript. In their rebuttal, the authors have adequately addressed this reviewer's concerns by adding additional experiments and providing reasonable arguments in the case of the minor concern. Thus, this reviewer has no further concerns and would be amenable to acceptance and publication.

Referee #3:

Authors have followed the considerations of the reviews requested, both from myself and the other reviewer. Authors have thoroughly included pertinent previous literature to bolster the significance of their work in the context of current knowledge. Further, by including Proximity Ligation Assay analyses, authors have provided novel data to help our understanding of how these novel protein-protein interactions are occurring in situ within fixed cells, extending western blot analyses. Immunofluorescence studies with these proteins strongly validates the biochemical analyses and western blots. Authors have included new models that greatly facilitates the ease of readership for a broad audience. The physiological relevance of this new link between Smad4 and its phosphatase

Wip1 is strongly supported by in vivo studies using Xenopus models and in cultured cancer cell lines. Given the fundamental role for Smad4 in development, cancer, and fibrosis, I strongly support the publication of this work in EMBO. In sum, I recommend that Editors accept this manuscript for publication as it stands.

Sincerely,

Lauren Albrecht

2nd Revision - authors' response 30 January 2020

We have made all editorial changes you requested in our final revised manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sun-Cheol Choi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48693

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were determined based on standard protocols in the field and our previous experiments. Each experiment was repeated at least two or three times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Based on our previous experiences, the number of Xenopus embryos chosen as a good sample size is as follows: 100-200 embryos for phenotypic analysis and 30-50 embryos for in situ hybridization experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Allocation of animals or samples was random.
For animal studies, include a statement about randomization even if no randomization was used.	Eggs from a female frog were in vitro fertilized and then allocated randomly for injection experiments.
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.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We used t-test to assess it.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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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/

Is there an estimate of variation within each group of data?	Yes. Data are presented as the mean ± SEM.
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).	Primary antibodies used for IPs, PLA and western blot analysis are as follows: anti-Smad1 (for IP, Santa Cruz, sc-7965), anti-Smad1 (Cell signaling, #6944), anti-P-Smad1/5 (Cell signaling, #9516), anti-Smad2 (for IP, Santa Cruz, sc-101153), anti-Smad2 (Cell signaling, #5339), anti-P-Smad2 (Cell signaling, #3108), anti-Smad4 (for IP and PLA, Santa Cruz, sc-7966), anti-Smad4 (Cell signaling
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MDA-MB231 and MDA-MB468 cells were a gift from Suhwan Chang (University of Ulsan College of Medicine) and HEK293T cells were a gift from Hun Sik Kim (University of Ulsan College of Medicine). HeLa cells were obtained from Jin-Kwan Han (POSTECH). All cell lines were tested and free of 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.	Male and female wild-type Xenopus laevis frogs (purchased from NASCO & Korean Xenopus Resource Center for Research) were raised and maintained at 18-20°C using standard methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This study was compliant with all relevant ethical regulations regarding animal research. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences, Asan Medical Center.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	This study was performed in compliance with the ARRIVE guidelines.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	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. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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