pVHL-mediated SMAD3 degradation suppresses TGFß signalling

Jun Zhou, Yasamin Dabiri, Rodrigo Gama-Brambila, Shahrouz Ghafoory, Mukaddes Altinbay, Arianeb Mehrabi, Mohammad Golriz, Biljana Blagojevic, Stefanie Reuter, Kang Han, Anna Seidel, Ivan Dikic, Stefan Wölfl, and Xinlai Cheng

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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.)

February 8, 2021

Re: JCB manuscript #202012097

Dr. Xinlai Cheng
Goethe University Frankfurt
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Dear Dr. Cheng,

Thank you for submitting your manuscript entitled "pVHL-mediated SMAD2/3 degradation supresses TGFß signalling". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

While both reviewers comment favorably and recommend further consideration of this work for publication in JCB, they both raised concerns that would need to be addressed in a revised version. The reviewers points all seem reasonable to try to address. Some of the important concerns require text revisions (e.g. a clear mention of other Smad ubiquitination mediators and their regulatory roles in TGFb and BMP signaling, both in the Introduction and the Discussion sections). Others will require new experimental work in order to increase the rigor and impact of this manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data.

Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Joan Massague
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Zhou and colleagues present an interesting paper on a novel regulator of TGFβ signaling that acts at the level of SMAD3. This is the well-established ubiquitin ligase VHL, best known for its involvement in the hypoxic control of gene expression via the transcription factor HIF1α. Unbiased screening for proteins that bind to a chemical compound (E738), previously established by the same authors to impact on SMAD2/SMAD3 protein stability, identified pVHL as a E738 and SMAD3-interacting protein. pVHL is shown to ubiquitylate and degrade proteasomally SMAD3 and specific key amino acid residues important for the interaction and/or ubiquitylation reaction have been identified. Overall, pVHL is shown to act as a negative regulator of TGFβ signaling in human cell models and in Drosophila wing imaginal disc differentiation.

The paper presents a serious number of complementary and convincing experiments. The biochemical/mechanistic part of the paper is rather strong, yet the biological part is slightly weaker, but convincing. I therefore have no major comment and I find this paper as providing an important new element in the understanding of TGFβ/SMAD signaling. Yet, I point to several minor points

aiming at increasing the clarity and significance of the presented work, which I think would be useful if the authors tried to cover constructively in their paper.

Specific comments listed in figure order (most crucial points):
1. The title emphasizes SMAD2/3, yet most mechanistic studies address SMAD3. As repeated below, some more emphasis on SMAD2 (also in view of the Drosophila work) is warranted.
2. The paper conveys the notion that SMAD3 is hydroxylated via a PHD protein on P403. To the best of my understanding, this has not been demonstrated experimentally, despite the use of CoCl2 that inhibits prolyl-hydroxylation events in cells. This point, if demonstrated, would raise the novelty of the paper seriously. However, if the authors do not want to demonstrate SMAD3 hydroxylation on P403, the relevant text can be corrected to relay the exact experimental findings.
3. The results claim even in one of the titles that pVHL interacts with SMAD3 via the MH2 domain. To the best of my understanding, this is not shown. In fact, Figure S3A shows that SMAD3C is not affected by pVHL OE or KD. Accordingly, the authors write that the linker domain contributes to the effect of pVHL on SMAD3. It would therefore be nice to demonstrate, using protein-protein interaction (not only protein expression, Figure S3A) assays, the domains of SMAD3 required for the interaction.
4. Related to the previous comment, some detail generates confusion. The results describe that SMAD3 degradation was considerably attenuated in the case of MH1-only variant SMAD3C (Fig. 3B and S3A). If I am not mistaken SMAD3C is the MH2-only construct, right? Figure S3A: lacks the pVHL immunoblot controls and, is there some mislabelling of the lanes in the SMAD3EPSM construct? The immunoblots of this figure would be nice to be complemented with co-IP SMAD3/pVHL experiments at least for 1-2 important SMAD3 constructs.
5. Figure 3C: The LH/NGPL motif is conserved among all SMADs (SMAD1-5, 8 but not in SMAD7, less in SMAD6) from human to Drosophila and C. elegans. It would be nice to show this in a sequence conservation chart especially since Drosophila is used as a biological model. The motif lies at the junction of beta sheet 11 and alpha helix 5 of the MH2 domain. It will be exciting if the authors can provide some structural comparison between the SMAD motif and the HIF1α motif.
6. Figure 3C: Lysine 378 is part of the KGWG motif that is conserved among all SMADs (SMAD1-7) in all species analyzed so far. Ubiquitylation of K507 in SMAD4 has been previously demonstrated by Morén A, et al. (Differential ubiquitination defines the functional status of the tumor suppressor Smad4. J Biol Chem. 2003;278(35):33571-82), and then called "universal ubiquitylation site" in all SMADs by Dupont S, et al. (FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell. 2009;136(1):123-35). Since the paper starts by analyzing both SMAD2 and SMAD3, and based on the conservation of K378 among SMADs, it is worth discussing possible conservation of the mechanism in the SMAD family.
7. The Drosophila gene CG9008 (Hevia et al, 2017) is used as a readout of Drosophila TGFβ signaling. Does this gene encode for a fly Glucose-6-phosphate 1-epimerase? It would be nice for the non-specialist to explain the function of this gene and its contribution to fly wing cell proliferation.
8. The discussion of this paper is very nice. Yet, I missed a thorough discussion on alternative mechanisms of SMAD2 and SMAD3 protein ubiquitylation by different ubiquitin ligases. Such a comparative discussion could also raise some speculation as to the special functions pVHL may have in regulating TGFβ signaling relative to the other SMAD degradation mechanisms.

More detailed points (not as crucial but important for the trustworthiness of the details):
9. Semantics: please number text pages and figures!
10. Figure S1E: the impact of the genetic perturbations on SMAD3/3 protein levels are borderline observable by a naked eye. Quantification of such immunoblots is needed. Similarly, whereas the results of Figure 1A are convincing, the repeat of the same experiment in Figure 4A shows no effect

of pVHL on SMAD2/3 or pSMAD2/3. The assay is tricky and does not work every time and for this reason requires quantification.

11. Figure S1I: the immunohistochemical signals of pVHL and SMAD3 are over-saturated. Please provide lower exposure images.

12. Figure 2B lacks the FLAG-SMAD3 without pVHL co-expression control.

13. Figure 2C: the FLAG-SMAD3 ubiquitylation patterns are evident but the impact of pVHL is a bit hard to visualize. This will improve if the gel electrophoresis is run longer time and the high MW smears resolve better.

14. Figure 2E: ATP concentration and immunoblotting is described as the assay performed. I could not find the relevant immunoblotting data. Related, Figure 3D: ubiquitylation assay using ATP level analysis at least for this figure should be complemented with immunoprecipitation-immunoblot as done for Figure 2C.

15. Figure 4B, luciferase reporter: the controls without pVHL OE are missing.

16. Figure 4C, S4B: ID1 and ID2 RT-PCR: in which cells and for how long was the TGFβ stimulation? ID1 and ID2 are also known to be downregulated by TGFβ (best mechanistic evidence for this provided by the Massagué lab). So, explaining the conditions of the experiments is important.

17. Figure 5: the CG9008 ISH is important to show after Babo RNAi and SMAD2-RNAi as shown in the graph 5N. This aims at understanding whether the Baboon, SMAD2 pathway is active or not during normal wing disc proliferation, and whether the CG9008 gene is directly downstream of SMAD2.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Zhou et al. report that pVHL acts as an E3 ligase mediating SMAD2/3 ubiquitination and degradation for inhibiting TGFβ signaling. In particular, the study has provided compelling evidence to reveal that pVHL physically interacts with SMAD3 and mediates its ubiquitination and proteasomal degradation. Importantly, the authors also identified key protein domains and motifs on both proteins that are crucially involved in pVHL-mediated SMAD degradation, such as the LxxP motif located in the MH2 domain of SMAD3. Besides the mechanistic part, the study investigated the pVHL-SMAD axis in the context of TGFβ-mediated cell migration and Drosophila's wing development, of which the associated phenotypes are compatible with the mechanistic discoveries. By identifying pVHL as a new regulator for SMAD2/3, the study appears to provide novel mechanistic insights for understanding TGFβ signaling. However, several major and minor concerns listed below need to be further explored and clarified.

1. Ubiquitin-dependent degradation is known to play crucial roles in regulating TGFβ signaling. For example, E3 ubiquitin-protein ligase Nedd4l can target activated Smad2/3 for degradation. Accordingly, what will be the circumstance in which SMAD2/3's ubiquitin-dependent degradation is primarily mediated by pVHL? Also, some introduction of the previous study on SMAD ubiquitination should be added to the text.

2. Regarding the LxxP motif located in the MH2 domain of SMAD3 responsible for pVHL's recognition, is it well conserved among all R-SMADs? Does this motif also exist in the MH2 domain of I-SMADs and Co-SMAD?

3. For the in vivo probe of Drosophila wing growth and patterning, mutant form of dSMAD2, which cannot be recognized by dVHL, should be created and tested to explore whether its expression can compromise the phenotype caused by dVHL overexpression.

4. The expression pattern of dSMAD2 should be examined upon manipulating dVHL expression in Drosophila.

5. In Fig. 4A, it appears that pVHL knockdown does not alter the protein level of SMAD2/3. An explanation & clarification is needed.

6. Authors indicated that a patient sample was used for staining in Fig. 1E. Was this from the normal tissue or cancer tissue? Also, the patient information such as cancer type (HCC or metastasis to the liver) should be described.

7. Human samples have been stained for pVHL and SMAD2/3. Is the staining pattern similar or different when comparing normal and cancer tissues?

8. Scale bars were missing in several photos, such as the ones in Fig. 5.

9. The Authors aimed to show linker domain can also promote the SMAD3 degradation, besides the MH2 domain, and thus they created the MH2-only SMAD3C variant. However, the authors stated SMAD3C is the MH1-only variant, which is not correct.

Journal of Cell Biology #202012097

Response to Reviewers

We thank the reviewers for the helpful comments and suggestions. We have outlined below how we addressed them by additional experiments and clarifications in the manuscript.

Reviewer #1 (Comments to the Authors (Required)):

Zhou and colleagues present an interesting paper on a novel regulator of TGFβ signaling that acts at the level of SMAD3. This is the well-established ubiquitin ligase VHL, best known for its involvement in the hypoxic control of gene expression via the transcription factor HIF1α. Unbiased screening for proteins that bind to a chemical compound (E738), previously established by the same authors to impact on SMAD2/SMAD3 protein stability, identified pVHL as a E738 and SMAD3-interacting protein. pVHL is shown to ubiquitylate and degrade proteasomally SMAD3 and specific key amino acid residues important for the interaction and/or ubiquitylation reaction have been identified. Overall, pVHL is shown to act as a negative regulator of TGFβ signaling in human cell models and in Drosophila wing imaginal disc differentiation.

The paper presents a serious number of complementary and convincing experiments. The biochemical/mechanistic part of the paper is rather strong, yet the biological part is slightly weaker, but convincing. I therefore have no major comment and I find this paper as providing an important new element in the understanding of TGFβ/SMAD signaling. Yet, I point to several minor points aiming at increasing the clarity and significance of the presented work, which I think would be useful if the authors tried to cover constructively in their paper.

Answer: We would like to thank the reviewer for the supportive and helpful comments.

Specific comments listed in figure order (most crucial points):
1. The title emphasizes SMAD2/3, yet most mechanistic studies address SMAD3. As repeated below, some more emphasis on SMAD2 (also in view of the Drosophila work) is warranted.

Answer: We agree with the reviewer that our manuscript is mostly address the role of VHL on SMAD3. Our initial screen and results identified VHL interfere with SMAD2/3 to promote its degradation using an antibody against both SMAD2 and 3 (Figure 1). The mechanistic investigation on how VHL interacts with and regulates R-SMAD is focused on SMAD3 in human cells. In addition, the TGFß/SMAD signaling in *Drosophila* contains only one

regulatory SMAD called Smox and it is closely related to SMAD2 and SMAD3 in vertebrates (Peterson and O'Connor, 2014). We therefore changed the title of the manuscript to "pVHL-mediated SMAD3 degradation suppresses TGFß signalling". However, because of highly concerved LxLxxP motif cross R-SMADs, we postulate that pVHL is an E3 ligase for all members of R-SMADs, including SMAD2. We discussed it in the revision.

2. The paper conveys the notion that SMAD3 is hydroxylated via a PHD protein on P403. To the best of my understanding, this has not been demonstrated experimentally, despite the use of CoCl2 that inhibits prolyl-hydroxylation events in cells. This point, if demonstrated, would raise the novelty of the paper seriously. However, if the authors do not want to demonstrate SMAD3 hydroxylation on P403, the relevant text can be corrected to relay the exact experimental findings.

Answer: We thank the reviewer for raising this concern. To address this, we have performed additional experiments to respectively immunoprecipitate FLAG-SMAD3FL (Full length), FLAG-SMAD3AAAA, FLAG-SMAD3K-R and FLAG-SMAD3N (MH1 only). We detected hydroxylated proline with specific hydroxyproline antibody (Abcam, #ab37067) only in FLAG-SMAD3FL and FLAG-SMAD3K-R, suggesting that P403 is hydroxylated. We included this new result in the revised manuscript (New Fig. 3G, please also see it below).
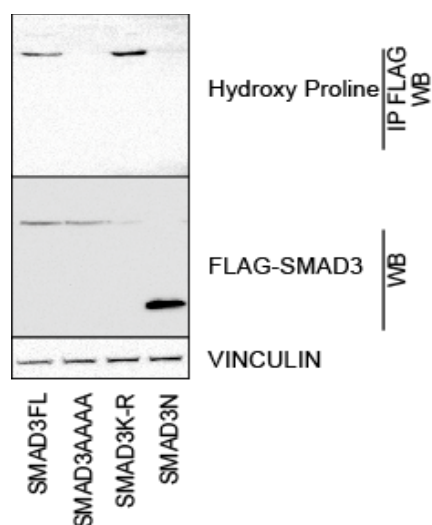

New Fig. 3G: P403 is hydroxylated. HeLa cells were respectively transfected with FLAG-SMAD3FL (Full length), FLAG-SMAD3AAAA, FLAG-SMAD3K-R and FLAG-SMAD3N (MH1 only). FLAG-SMAD variants were immunoprecipitated with FLAG antibody. Hydroxy Proline antibody was used to detect the hydroxylated proline.

3. The results claim even in one of the titles that pVHL interacts with SMAD3 via the MH2 domain. To the best of my understanding, this is not shown. In fact, Figure S3A shows that SMAD3C is not affected by pVHL OE or KD. Accordingly, the authors write that the linker domain contributes to the effect of pVHL on SMAD3. It would therefore be nice to demonstrate, using protein-protein interaction (not only protein expression, Figure S3A) assays, the domains of SMAD3 required for the interaction.

Answer: Our quantified result from three independent experiments show that more than 1.5-fold higher expression of FLAG-SMAD3C (New **Fig. 3B**). We replaced New **Fig. S3A** in our original manuscript with a better image (shown below Fig. S3A). Moreover, according to the Reviewer1's suggestion, we investigated the binding affinity of pVHL to five SMAD3 variants, which confirmed that C-terminal of SMAD3 is indispensable for the binding of pVHL to SMAD3. The linker domain is most likely also participated in the interaction, because FLAG-SMAD3LC was more sensitive to pVHL than FLAG-SMAD3C (New **Fig. S3B**, please also see it below). However, the function of the linker domain in this context needs be further investigated. Thus, we removed 'the linker domain contributes to the effect of pVHL on SMAD3' in the revision.
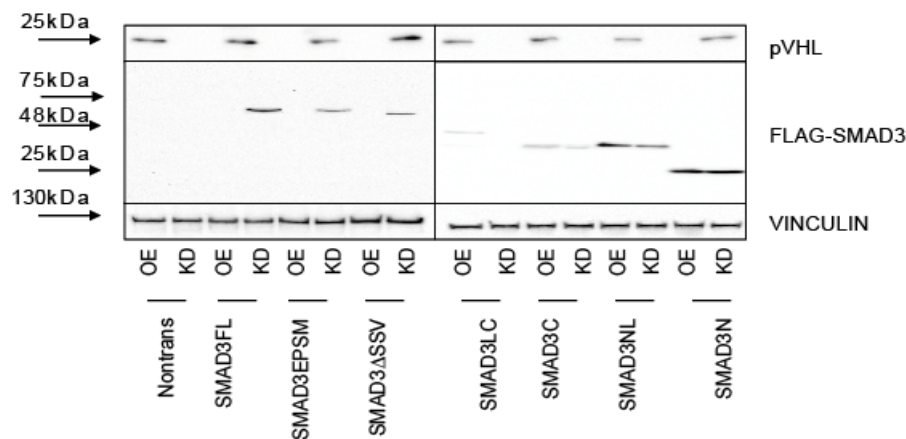
New Fig. S3A: The influence of pVHL knockdown or overexpression on FLAG-SMAD3 variants. OE: pVHL WT overexpression; KD: siVHL knockdown; SMAD3FL: full length FLAG-SMAD3; SMAD3EPSM: FLAG-SMAD3 carrying 4 mutations in linker region; SMAD3LC: FLAG-SMAD3 only with linker (L) and MH2 (C: C-terminal); SMAD3C: FLAG-SMAD3 only with MH2; SMAD3NL: FLAG-SMAD3 only with MH1 (N-terminal) and linker; SMAD3N: FLAG-SMAD3 only with MH1 and SMAD3ΔSSV: FLAG-SMAD3 without SSV at C-terminal for active phosphorylation.
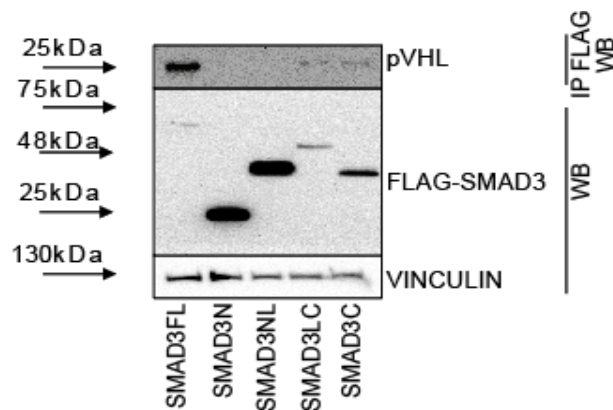
New Fig. S3B: C-terminal is required for SMAD3 interacting with pVHL. HeLa cells were transfected with variants of FLAG-SMAD3 (FLAG-SMAD3FL, FLAG-SMAD3N, FLAG-SMAD3NL, FLAG-SMAD3LC and FLAG-SMAD3C). FLAG-SMAD3s were immunoprecipitated and pVHL antibody was used to detect the presence of pVHL on FLAG-SMAD3s.

4. Related to the previous comment, some detail generates confusion. The results describe that SMAD3 degradation was considerably attenuated in the case of MH1-only variant SMAD3C (Fig. 3B and S3A). If I am not mistaken SMAD3C is the MH2-only construct, right? Figure S3A: lacks the pVHL immunoblot controls and, is there some mislabelling of the lanes in the SMAD3EPSM construct? The immunoblots of this figure would be nice to be complemented with co-IP SMAD3/pVHL experiments at least for 1-2 important SMAD3 constructs.

Answer: We thank the reviewer for notifying this typo. Indeed, FALG-SMAD3C contains only MH2 domain (199-425). In the revised manuscript, we added pVHL and replaced it with a better resolution WB image (please see the above Fig. S3A). According to the Reviewer1's suggestion, we also performed Co-IP of FLAG-SMAD3/pVHL experiments. The result sustained our finding that MH2 domain (C-terminal) is indispensible for the interaction of pVHL with SMAD3 (please see the above Fig. S3B).

5. Figure 3C: The LH/NGPL motif is conserved among all SMADs (SMAD1-5, 8 but not in SMAD7, less in SMAD6) from human to Drosophila and C. elegans. It would be nice to show this in a sequence conservation chart especially since Drosophila is used as a biological model. The motif lies at the junction of beta sheet 11 and alpha helix 5 of the MH2 domain. It will be exciting if the authors can provide some structural comparison between the SMAD motif and the HIF1α motif.

Conserved sequences in Smad-protein MH2-domain C-termini

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name          accession       AA-position          fragment sequences                              AA-position

structure elements (hSmad3)  [sheet]    [helix] [sheet]  [helix]                                    (P)(P) activating phosphorylation

R-Smad                                      hSmad3 K378
dmeSMOX       AAC83344.1      433   MSFVKGWG-AEYRRQTVTSTPCWIELHLNGPLQWLDRVLTQMGSPRLPCSSMS    484
dmeMAD        AAB60230.1      404   MSFVKGWG-AEYFRQDVTSTPCWIETHLHGPLQWLDKVLTQMGSPHNADSSVS    455

hSMAD3        P84022.1        374   MSFVKGWG-AEYRRQTVTSTPCWIELHLNGPLQWLDKVLTQMGSPSIRCSSVS    425
hSMAD2        Q15796.1        416   MSFVKGWG-AEYRRQTVTSTPCWIELHLNGPLQWLDKVLTQMGSPSVRCSSMS    467
hSMAD5        Q99717.1        414   MSFVKGWG-AEYFRQDVTSTPCWIETHLHGPLQWLDKVLTQMGSPLYPTSSVS    465
hSMAD1        Q15797.1        414   MSFVKGWG-AEYFRQDVTSTPCWIETHLHGPLQWLDKVLTQMGSPHNPTSSVS    465
hSMAD9        O15198.1        416   MSFVKGWG-AEYFRQDVTSTPCWIETHLHGPLQWLDKVLTQMGSPHNPTSSVS    467

dreSMAD3      NP_571646.1     374   MSFVKGWG-AEYRRQTVTSTPCWIELHLNGPLQWLDKVLTQMGSPSIRCSSVS    425
dreSMAD2      XP_005155615.1  413   MSFVKGWG-AEYRRQTVTSTPCWIELHLNGPLQWLDKVLTQMGSPSVRCSSMS    464
dreSMAD5      XP_005173221.1  413   MSFVKGWG-AEYFRQDVTSTPCWIEVHLHGPLQWLDKVLTQMGSPLYPTSSVS    464
dreSMAD9      XP_021334835.1  415   MSFVKGWG-AEYFRQDVTSTPCWIETHLHGPLQWLDKVLTQMGSPHNPTSSVS    466

Co-Smad
dmeMedea      NP_524610.1     725   LSFVKGWG-PDYPRQSIKETPCWIEVHLHRALQLLDEVLHAM--PIDGPRAAA    771

hSMAD4        Q13485.1        503   MSFVKGWG-PDYPRQSIKETPCWIEIHLHRALQLLDEVLHTM--PIADPQPLD    552

dreSMAD4      NP_001116172.1  498   MSFVKGWG-PDYPRQSIKETPCWIEIHMHRALQLLDEVLHTM--PIADPTPLD    547

I-Smad:
dmeDAD        ALC47700.1      570   ISFYKGWGyNQYRRQDIMGCPCWLEVHFNHLR--------------------    601

hSMAD6        O43541.2        466   ISFAKGWG-PCYSRQFITSCPCWLEILLNNPR--------------------    496
hSMAD7        O15105.1        397   ISFVKGWG-QCYTRQFISSCPCWLEVIFNSR---------------------    426

dreSMAD6      AAI39622.1      456   ISFAKGWG-PCYSRQFITSCPCWLEILLNNHR--------------------    486
dreSMAD7      NP_778257.2     343   ISFVKGWG-QCYTRQFISSCPCWLEVIFNNR---------------------    372
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Answer: As suggested by the reviewer, we now included R-SMAD homologous in Drosophila (dmeSMOX and dmeMAD) and Zebrafish (dreSMAD2, dreSMAD3, dreSMAD5 and dreSMAD9), as well as SMAD4 and I-SMAD homologous in our new alignment analysis. The result confirmed that LxLxxP is highly conserved in all R-SMAD variants, but not in co-SMADs or I-SMADs. We added this new result in New **Fig. S3C** in the revised manuscript. A recently reported from Miyazono et al (Miyazono Science Signaling, 2018) showed the interaction of SKI with helix bundle region of SMAD2. P445 was included in this region and is equal to P402 of hSMAD3, suggesting the potential contribution of P402 to the interaction of SMAD2/3 with other proteins, for instance pVHL. However, additional experiments need to be done for understanding of the binding model of SMAD3 and pVHL. We discussed it in the revision (Page 8 and 12). We tried, but could perform the structure comparison of binding motif between HIF1α and SMAD3 because of lack of the specific structure biology background. We are looking for cooperation and will complete it in our future research.

6. Figure 3C: Lysine 378 is part of the KGWG motif that is conserved among all SMADs (SMAD1-7) in all species analyzed so far. Ubiquitylation of K507 in SMAD4 has been previously demonstrated by Morén A, et al. (Differential ubiquitination defines the functional

status of the tumor suppressor Smad4. J Biol Chem. 2003;278(35):33571-82), and then called "universal ubiquitylation site" in all SMADs by Dupont S, et al. (FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell. 2009;136(1):123-35). Since the paper starts by analyzing both SMAD2 and SMAD3, and based on the conservation of K378 among SMADs, it is worth discussing possible conservation of the mechanism in the SMAD family.

Answer: We thank the reviewer for her/his suggestion. We now discussed the mentioned literatures in the revised manuscript (Page 12).

7. The Drosophila gene CG9008 (Hevia et al, 2017) is used as a readout of Drosophila TGFβ signaling. Does this gene encode for a fly Glucose-6-phosphate 1-epimerase? It would be nice for the non-specialist to explain the function of this gene and its contribution to fly wing cell proliferation.

Answer: We thank the reviewer for her/his suggestion. We have now discussed the function of CG9008 and its contribution to fly wing development in the Discussion section in the revised manuscript (please see page 14).

8. The discussion of this paper is very nice. Yet, I missed a thorough discussion on alternative mechanisms of SMAD2 and SMAD3 protein ubiquitylation by different ubiquitin ligases. Such a comparative discussion could also raise some speculation as to the special functions pVHL may have in regulating TGFβ signaling relative to the other SMAD degradation mechanisms.

Answer: We agree with the reviewer that there are various ubiquitin ligases/deubiquitinating enzymes known to be involved in the regulation of the TGFβ pathway. We have now introduced and comparatively discussed the role of these known ligases (e.g., NEDD4L and Smurf2) in the revised manuscript, which could put the VHL-mediated regulation of total Smad2/3 levels in a better context. (please see page 3 and page 12).

More detailed points (not as crucial but important for the trustworthiness of the details): 9. Semantics: please number text pages and figures!

Answer: We have now added the page number on the main text and figures in the revised manuscript.

10. Figure S1E: the impact of the genetic perturbations on SMAD3/3 protein levels are borderline observable by a naked eye. Quantification of such immunoblots is needed. Similarly,

whereas the results of Figure 1A are convincing, the repeat of the same experiment in Figure 4A shows no effect of pVHL on SMAD2/3 or pSMAD2/3. The assay is tricky and does not work every time and for this reason requires quantification.

Answer: We agreed with the reviewer for this point. The quantified WB data of Figure S1E is shown in **Fig. 1B** in our original manuscript (also shown below). Regarding **Fig. 4A**, we now added quantified WB data of SMAD2/3 in **New Fig. S4A** in the revised manuscript. These results from three independent experiments demonstrate that the protein level of SMAD2/3 and pSMAD2/3 are negatively correlated with the expression of pVHL. Please also see the WB and quantification in New **Fig. 4A** and New **Fig. S4A** below.
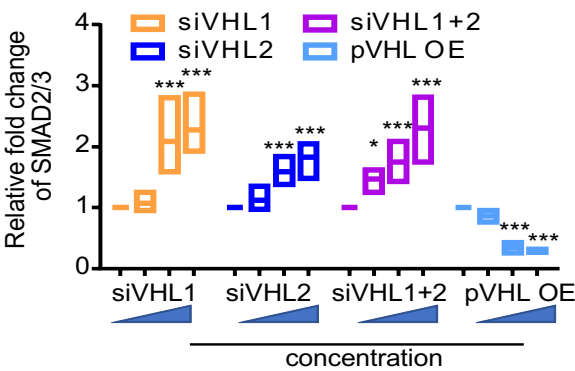

Fig. 1B: SMAD2/3 stability is negatively related to the expression of pVHL. The correlation of pVHL to SMAD2/3 expression was quantified by densitometric analysis. siVHL1, siVHL2 and combined siVHL1+2 were used to study SMAD2/3 stability in pVHL KD cells. pVHL OE indicates cells overexpressing pVHL. Three concentrations of either siRNAs or DNA were used. The immunoblotting results can be found in **Fig. S1E** and **Fig. S1F**. The relative SMAD2/3 expression over respective control was depicted. One-way ANOVA was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; lower and upper ends of bars respectively indicate the minimum and maximum values and the centre presents the median.
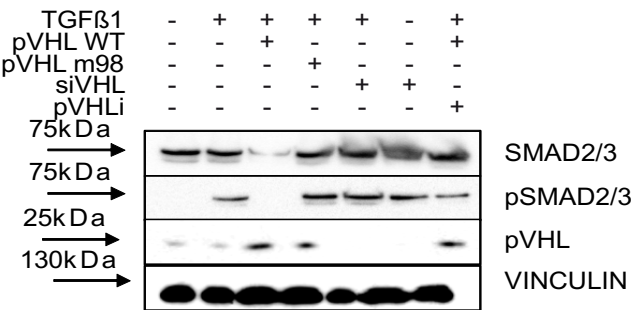

New Fig. 4A: pVHL represses the phosphorylation of SMAD2/3 with stimuli of TGFβ. The phosphorylation of SMAD2/3 was compared in cells expressing pVHL WT or pVHL m98, or pVHL-deficient cells, or cells treated with pVHL inhibitor. Densitometric analysis can be found in **Fig. S4A.**
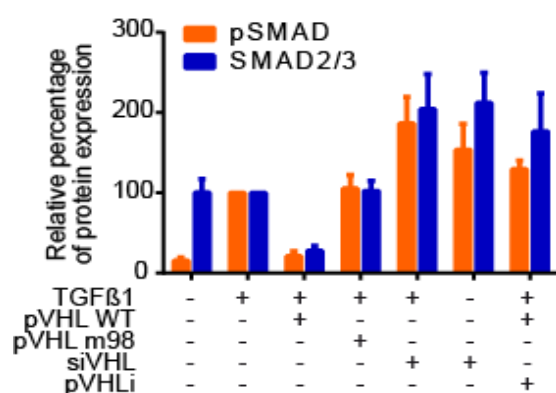
New Fig. S4A : Densitometric immunoblotting result of SMAD2/3 and pSMAD2/3

11. Figure S1I: the immunohistochemical signals of pVHL and SMAD3 are over-saturated. Please provide lower exposure images.

Answer: As the reviewer suggested, we provide lower exposure images in **New Fig. S1I** (please also see it below).
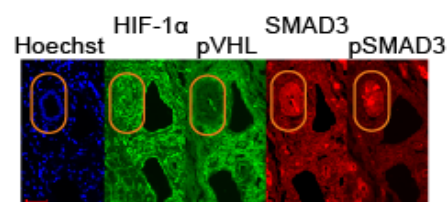
New Fig. S1I : pVHL is negatively correlated to SMAD3 and phospho-SMAD3 expression in patient tissues. No correlation was found between HIF-1α and SMAD3.

12. Figure 2B lacks the FLAG-SMAD3 without pVHL co-expression control.

**Answer**: As the reviewer suggested, we now added images from cells expressing SMAD-OFP without pVHL-GFP as control in the **New Fig. 2B** in the revised manuscript (please also see the images below).
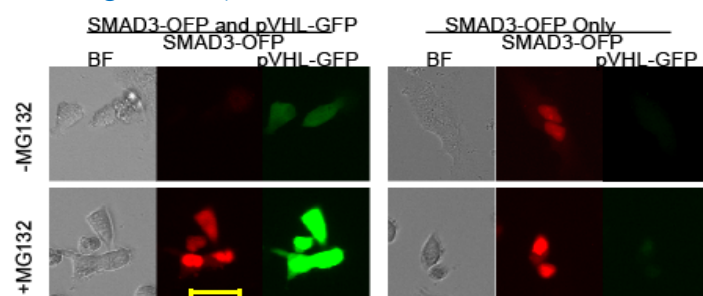
New Figure. 2B: MG132 rescued pVHL-mediated SMAD3 degradation, as shown by live cell imaging. Red: SMAD3-OFP; Green: VHL-GFP. Scale bar: 40 μm. Cells expressing only SMAD3-OFP were used as a control.

13. Figure 2C: the FLAG-SMAD3 ubiquitylation patterns are evident but the impact of pVHL is a bit hard to visualize. This will improve if the gel electrophoresis is run longer time and the high MW smears resolve better.
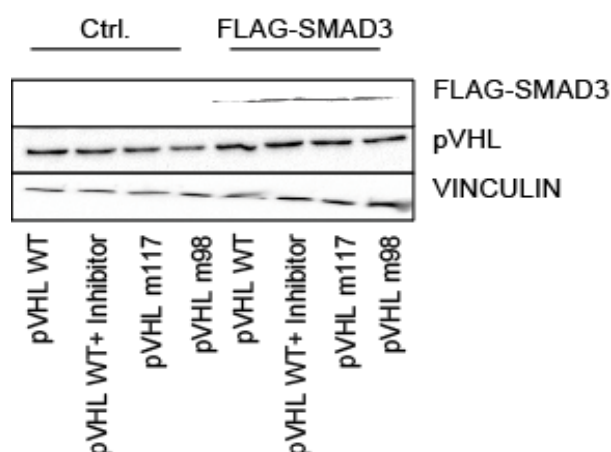
Answer: We take the reviewer's suggestion and re-run the gel electrophoresis. The **Fig. 2C** has now been replaced with the new images with better resolution.
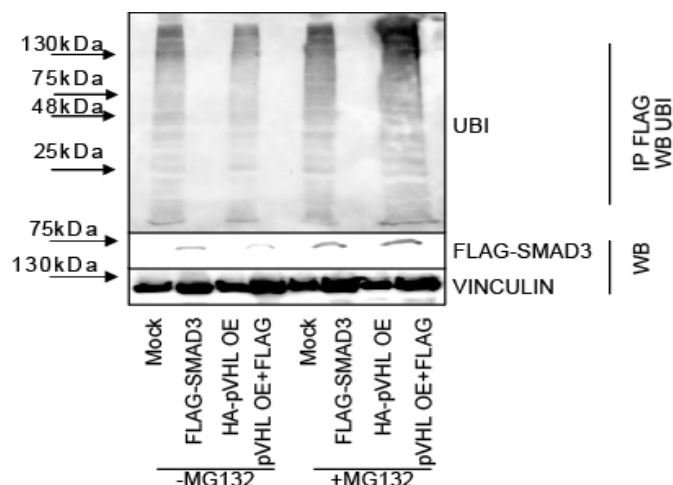
New Figure 2C: pVHL-mediated polyubiquitination on FLAG-SMAD3. The ubiquitination on SMAD3 was compared in the absence and presence of MG132 in cells expressing FLAG-SMAD3, HA-pVHL or the combination. FLAG-SMAD3 was precipitated and ubiquitination on FLAG-SMAD3 was detected with specific ubiquitin antibody.

14. Figure 2E: ATP concentration and immunoblotting is described as the assay performed. I could not find the relevant immunoblotting data. Related, Figure 3D: ubiquitylation assay using ATP level analysis at least for this figure should be complemented with immunoprecipitation-immunoblot as done for Figure 2C.

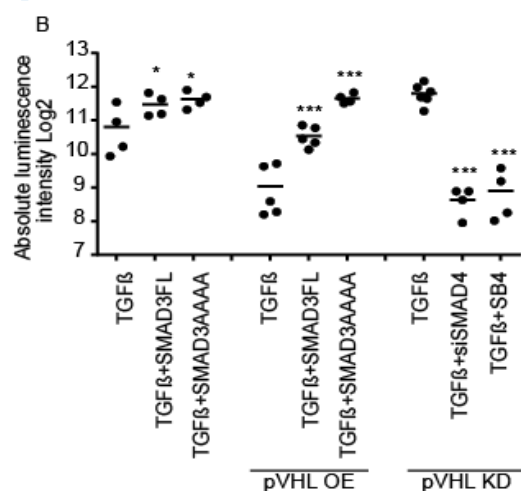**Answer**: We have now added the immunoblotting data, which confirmed that pVHL-mediated FLAG-SMAD3 degradation disappeared in the presence of pVHL inhibitor or using pVHL m117 or m98 variants (**New Fig. S2C**, please also see the results below).

Ctrl.   FLAG-SMAD3

FLAG-SMAD3
pVHL
VINCULIN

pVHL WT
pVHL WT+ Inhibitor
pVHL m117
pVHL m98
pVHL WT
pVHL WT+ Inhibitor
pVHL m117
pVHL m98

New Fig. S2C : in vitro ubiquitination assay analysis in immunoblotting.

15. Figure 4B, luciferase reporter: the controls without pVHL OE are missing.
Answer: We now added corresponding controls in **New Fig. 4B** in the revised manuscript
(please also see the results below).

B

Absolute luminescence intensity Log2

TGFß
TGFß+SMAD3FL
TGFß+SMAD3AAAA

TGFß
TGFß+SMAD3FL
TGFß+SMAD3AAAA

TGFß
TGFß +siSMAD4
TGFß+SB4

pVHL OE   pVHL KD

New Fig. 4B: pVHL interferes with TGFβ/SMAD3 signalling determined by TGFβ luciferase reporter. siSMAD4: SMAD4 siRNA; SB4: SB-431542, a TGFβ receptor inhibitor.

16. Figure 4C, S4B: ID1 and ID2 RT-PCR: in which cells and for how long was the TGFβ stimulation? ID1 and ID2 are also known to be downregulated by TGFβ (best mechanistic evidence for this provided by the Massagué lab). So, explaining the conditions of the experiments is important.

Answer: We have now added the detailed protocol in the supplemental materials. Briefly, HeLa cells were transfected with various plasmids as designed for 48 h. Serum starvation was performed for 4 h and treated with TGFß for 1 h with or without inhibitor. Total RNA was isolated with QIAzol (Qiagen, Germany).

17. Figure 5: the CG9008 ISH is important to show after Babo RNAi and SMAD2-RNAi as shown in the graph 5N. This aims at understanding whether the Baboon, SMAD2 pathway is active or not during normal wing disc proliferation, and whether the CG9008 gene is directly downstream of SMAD2.

Answer: We thank the reviewer for this comment. Previous studies have shown that knocking down Smox/dSMAD2 reduces the expression of CG9008 in the wing discs. Conversely, Smox overexpression enhances the CG9008 expression (Hevia et al., 2017). We now have mentioned these published data in the Results section in the revised manuscript (please see Page 10 and 11).

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Zhou et al. report that pVHL acts as an E3 ligase mediating SMAD2/3 ubiquitination and degradation for inhibiting TGFβ signaling. In particular, the study has provided compelling evidence to reveal that pVHL physically interacts with SMAD3 and mediates its ubiquitination and proteasomal degradation. Importantly, the authors also identified key protein domains and motifs on both proteins that are crucially involved in pVHL-mediated SMAD degradation, such as the LxxP motif located in the MH2 domain of SMAD3. Besides the mechanistic part, the study investigated the pVHL-SMAD axis in the context of TGFβ-mediated cell migration and Drosophila's wing development, of which the associated phenotypes are compatible with the mechanistic discoveries. By identifying pVHL as a new regulator for SMAD2/3, the study appears to provide novel mechanistic insights for understanding TGFβ signaling. However, several major and minor concerns listed below need to be further explored and clarified.

Answer: We thank the reviewer for her/his positive assessment.

1. Ubiquitin-dependent degradation is known to play crucial roles in regulating TGFβ signaling. For example, E3 ubiquitin-protein ligase Nedd4l can target activated Smad2/3 for degradation. Accordingly, what will be the circumstance in which SMAD2/3's ubiquitin-dependent degradation is primarily mediated by pVHL? Also, some introduction of the previous study on SMAD ubiquitination should be added to the text.

Answer: We thank the reviewer for this comment. As NEDD4L is the most prominent E3 ligase to regulate activated p-SMAD2/3 turnover, we found that pVHL mediates total SMAD3 degradation, whose levels could significantly impact the outcome of TGFß signaling pathway in cells and during *Drosophila* wing development. Importantly, the regulation of total (nonactivated/nonphosphorylated) R-Smads are a poorly investigated matter, as most of the literature have focused on the turnover of C-terminally phosphorylated R-Smads. Additionally, our new result showed that the hydroxylation at P402 might play an important role in Smad3 stability (**New Figure 3G**). Thus, the activity of PHD might be essential in this context. However, more detailed experiments need to be done in the future. We discussed it in the revision.

As suggested, we have also introduced different E3 ligases and their roles in the regulation of SMAD2/3 degradation in the revised manuscript (please see page 3 and page 12).

2. Regarding the LxxP motif located in the MH2 domain of SMAD3 responsible for pVHL's recognition, is it well conserved among all R-SMADs? Does this motif also exist in the MH2 domain of I-SMADs and Co-SMAD?

Answer: Yes, the LxLxxP is well conserved among all R-SMADs in human, Drosophila and Zebrafish. Please see our new alignment analysis in **New Fig. S3C** in the revised manuscript. The result from this new alignment analysis also showed that this motif does not exist in the MH2 domain of I-SMADs and Co-SMADs (**New Fig. S3C**).

3. For the in vivo probe of Drosophila wing growth and patterning, mutant form of dSMAD2, which cannot be recognized by dVHL, should be created and tested to explore whether its expression can compromise the phenotype caused by dVHL overexpression.

Answer: We agree with the reviewer that the in vivo validation of SMAD2-VHL interaction can strengthen our current model. We are generating the Drosophila transgenes expressing the mutated form of dSMAD2 (UAS-dSMAD2^{AAAA}), which are resistant to dVHL (work in progress). However, this requires a certain amount of additional time to obtain the transgenes and perform the requested experiments and we are not able to complete it during the period of revision because of corona pandemic. We thank the reviewer for her/his suggestion and will report the result in the near future.

In another aspect, we have shown that the tyrosine Y98 in the β-domain of pVHL is important for the interaction with SMAD2/3 for degradation in human cells (**New Fig. 2D**). In addition, expression of a mismatch VHL mutant (Y51H, equivalent to human residue Y98H) fails to reproduce the VHL gain of function or SMAD2 loss of function wing phenotypes (**New Fig. S5A-5H**). Taken together, these results suggest the interaction between SMAD2-VHL is required for the regulation of TGFβ signaling during Drosophila wing development.

4. The expression pattern of dSMAD2 should be examined upon manipulating dVHL expression in Drosophila.

Answer: As suggested by the reviewer, we performed phosphor-SMAD2/3 immunostaining in the Drosophila wing discs upon SMAD activation by either known down VHL using RNAi or expression of the active form of the TGFβ receptor (Babo^{Act}). We tested different human pSMAD2/3 antibodies such as pSMAD2 (Ser465/467) (138D4) (Cell signaling, Cat.No. 3108) and pSMAD2 (Ser465/467)/SMAD3 (Ser423/425) (D27F4) (Cell signaling, Cat.No. 8828).

We observed no signal with pSMAD2(Ser465/467) staining in the wing discs as shown in Revision Figure 1A-1C. Due to the high similarity of R-SMADs between TGFβ and BMP signaling, the pSMAD2 (Ser465/467)/SMAD3 (Ser423/425) antibody show cross-reactivity with the Drosophila homolog of BMP related SMAD1,5 and 8 (Mad). In Revision Figure 1D-1E, we saw a similar expression pattern of pSMAD2 (Ser465/467)/SMAD3 (Ser423/425) staining as pMad in the wing discs (Hufnagel et al., 2007), we therefore cannot rely on this antibody for pSmox staining in the Drosophila tissue.

We further requested the human pSMAD2 antibody generated by the laboratory of Peter Ten Dijke (Persson et al., 1998) and performed the staining in the Drosophila wing disc. However, the antibody only shows background staining and fails to detect pSMAD2 in the fly tissue as Smox RNAi does not reduce the signal of staining (**Revision Figure 1J**). Hence, the tested human pSMAD2/3 antibodies show either negative signal or false positive cross reactivity for detecting pSMAD2/3 activity in Drosophila tissues. Further work is needed to generate a Drosophila specific pSMAD2/3 antibody to study SMAD2 and SMAD3 activity.
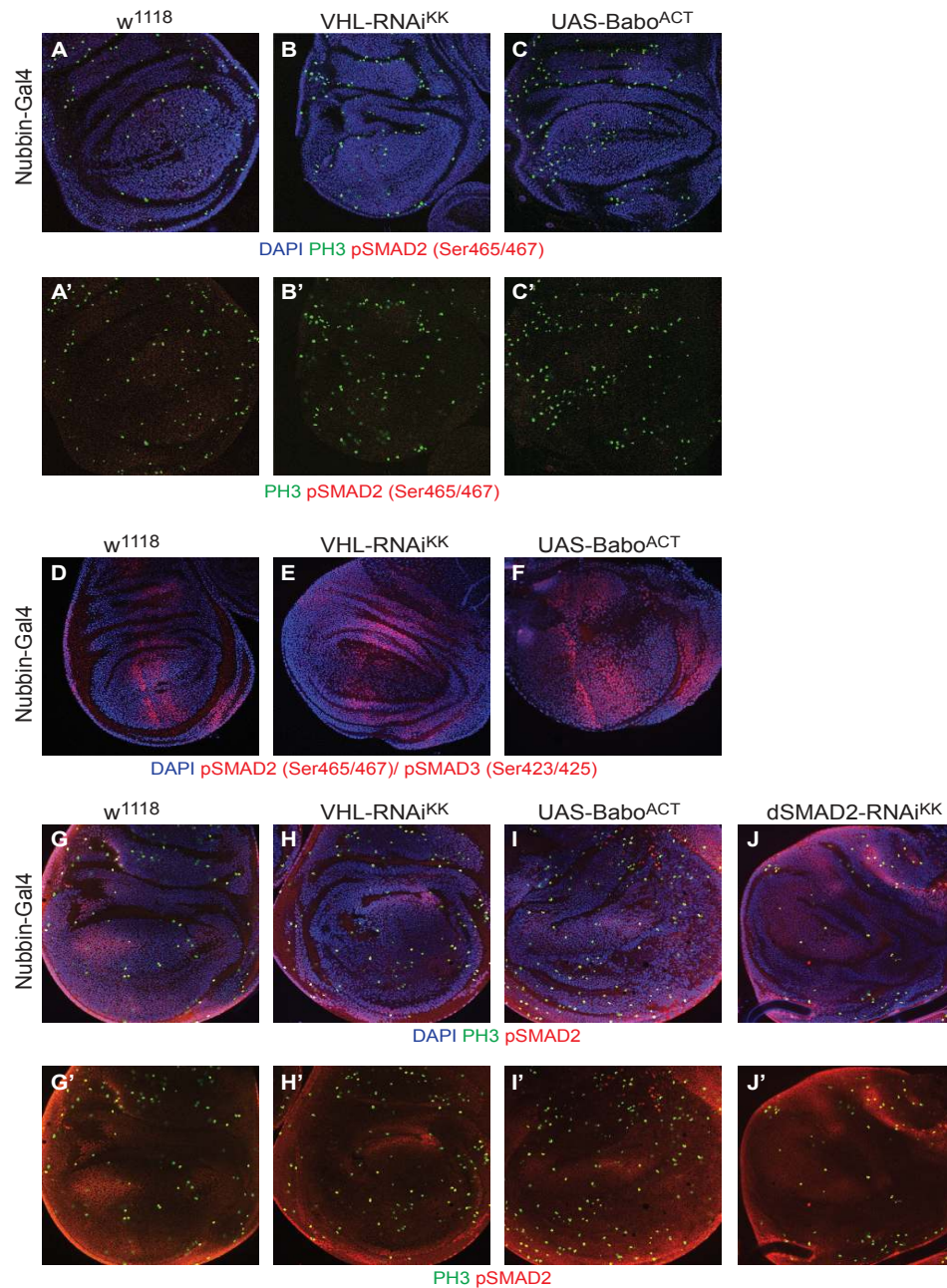
Revision Figure 1. Human pSMAD2/3 antibodies fail to detect pSMAD2/3 activity in Drosophila wing disc. (**A-C**) The representative image of human pSMAD2 (Ser465/467) antibody staining in Drosophila third instar wing imaginal disc with indicated genotypes. (**D-E**) The representative image of human pSMAD2 (Ser465/467)/pSMAD3 (Ser423/425) antibody staining in Drosophila third instar wing imaginal disc with indicated genotypes. (**G-J**) The representative image of human pSMAD2 antibody staining in Drosophila third instar wing imaginal disc with indicated genotypes. Nuclei are stained with DAPI in blue, PH3 positive cell are labelled in green, and pSMAD2/3 signal are labelled in red.

5. In Fig. 4A, it appears that pVHL knockdown does not alter the protein level of SMAD2/3. An explanation & clarification is needed.

Answer: Our quantified data from more than 3 independent experiments showed that expression of pVHL was negatively related to the protein level of SMAD2/3 (**New Fig. 1B** and **New Fig. S4A**). The influence of pVHL on SMAD2/3 is fluctuant because the transfection efficiencies are various in experiments. In the revised manuscript, we replaced a better representative image.

6. Authors indicated that a patient sample was used for staining in Fig. 1E. Was this from the normal tissue or cancer tissue? Also, the patient information such as cancer type (HCC or metastasis to the liver) should be described.

Answer: In **New Fig. 1E**, we used the cancer tissue from patient No. 7 (Liver metastasis from thyroid carcinoma). All information related to patient samples can be found in the section of Immunohistochemistry on patient samples of supplemental material.

7. Human samples have been stained for pVHL and SMAD2/3. Is the staining pattern similar or different when comparing normal and cancer tissues?

Answer: We found the inversed correlation between pVHL and SMAD2/3 in both non-cancerous (normal) and cancerous areas in the liver tissue from all 17 cancer patients. The detailed analysis can be found in **New Fig. S1H** in the revised manuscript.

8. Scale bars were missing in several photos, such as the ones in Fig. 5.

Answer: We thank the reviewer for noticing the missing scalebars. We have added the scale bars in **New Fig. 5** and **New Fig. S5** in the revised manuscript.

9. The Authors aimed to show linker domain can also promote the SMAD3 degradation, besides the MH2 domain, and thus they created the MH2-only SMAD3C variant. However, the authors stated SMAD3C is the MH1-only variant, which is not correct.

Answer: We thank the reviewer for this comment. We have now corrected it in the revised manuscript (Page 8). Moreover, we also precipitated FLAG-SMAD3 variants and investigated pVHL binding to FLAG-SMADs. We confirmed that MH2 domain is important for the pVHL-SMAD3 interaction (**New Fig. S3C**).
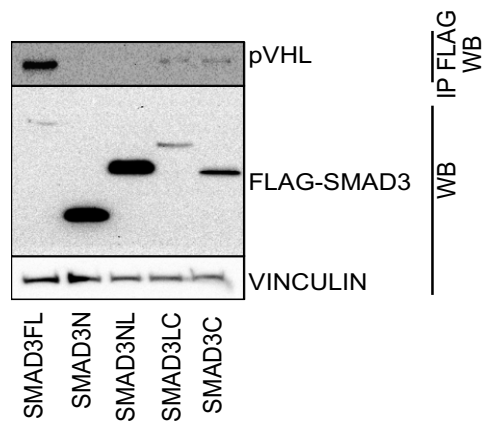
New Fig. S3C : C-terminal is required for SMAD3 interacting with pVHL. HeLa cells were transfected with variants of FLAG-SMAD3 (FLAG-SMAD3FL, FLAG-SMAD3N, FLAG-SMAD3NL, FLAG-SMAD3LC and FLAG-SMAD3C). FLAG-SMAD3s were immunoprecipitated and pVHL antibody was used to detect the presence of pVHL on FLAG-SMAD3s.

References

Hevia, C.F., López-Varea, A., Esteban, N., and Celis, J.F. de (2017). A Search for Genes Mediating the Growth-Promoting Function of TGFβ in the Drosophila melanogaster Wing Disc. Genetics 206, 231–249.

Hufnagel, L., Teleman, A.A., Rouault, H., Cohen, S.M., and Shraiman, B.I. (2007). On the mechanism of wing size determination in fly development. Proc. Natl. Acad. Sci. 104, 3835–3840.

Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engström, U., Heldin, C.H., Funa, K., and ten Dijke, P. (1998). The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. FEBS Lett. 434, 83–87.

July 8, 2021

RE: JCB Manuscript #202012097R

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Buchmann Institute for Molecular Life Sciences Pharmaceutical Chemistry Goethe-University
Frankfurt am Main/Pharmaceutical Chemistry
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Frankfurt am Main D-60438
Germany

Dear Dr. Cheng:

Thank you for submitting your revised manuscript entitled "pVHL-mediated SMAD3 degradation suppresses TGFß signalling". ". The reviewers now support publication so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, you must also attempt the remaining experiment suggested by reviewer #2.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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2) Figures limits: Articles may have up to 10 main text figures.

3) * Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis (e.g. 3G, S2C)

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so,

how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

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7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider

providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Joan Massague
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the revised paper, Zhou and colleagues clarify many of the ambiguities generated to this reviewer. The revised paper presents a strong account on the ubiquitin ligase VHL, which can regulate TGFβ signaling at the level of SMAD3. The combination of detailed biochemical assays with cell biological analysis convincingly show that pVHL is a SMAD3-interacting protein that ubiquitylates and degrades proteasomally SMAD3. This paper complements previous accounts on the mechanisms that regulate SMAD protein stability and also generates new interest regarding the potential co-regulation between oxygen sensing and TGF-beta signaling. I believe that this paper will make an important contribution to the multifaceted signaling by TGF-beta and to the prolific VHL field.

Reviewer #2 (Comments to the Authors (Required)):

Overall, the authors have satisfactorily addressed previously raised questions except for question #4.

In brief, my question aimed to explore if pVHL can mediate the degradation of SMAD2/3 in vivo. Based on the proposed working model, through ectopic expression of pVHL in the Drosophila, it is expected that less SMAD2/3 signal can be detected by immunostaining.

In the revised manuscript, the authors knocked down VHL, and then performed immunostaining on phosphorylated SMAD2/3. The staining results were inconclusive. The authors postulated that the antibody has cross-reactivity and might explain the negative results obtained.

Since the authors suggest that pVHL can mediate the degradation of total SMAD2/3 regardless of phosphorylation status and successfully performed immunostaining on total SMAD2/3 in Fig. 1E, it might be practical to conduct the immunostaining to explore if total SMAD2/3 are decreased upon dVHL overexpression in Drosophila.

pVHL-mediated SMAD3 degradation suppresses TGFß signalling

Journal of Cell Biology #202012097
Response to Reviewers

We would like to thank the editor as well as the reviewers for the useful comments and suggestions. We have indicated here how we have addressed the issues raised by reviewer#2 in detail. Additionally, we have carefully checked the manuscript in order to meet the formatting guidelines of the journal, as requested by the editor.

Dear Dr. Cheng:

Thank you for submitting your revised manuscript entitled "pVHL-mediated SMAD3 degradation suppresses TGFß signalling". The reviewers now support publication so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, you must also attempt the remaining experiment suggested by reviewer #2.

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Word count is approx. 6600 with characters: <40000.
2) Figures limits: Articles may have up to 10 main text figures.
There are 5 figures in total in the main text of our manuscript.
3) * Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis (e.g. 3G, S2C)
Scale bars are present in all microscopy images. We added molecular weight in all gel electrophoresis including 3G and S2C.
4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in

the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

We re-wrote the description of statistic analysis in figure legends and the materials and methods under the guidance of journal's instruction.

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

We modified the abstract under the guidance of journal's instruction.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

We double-checked and modified some parts of materials and methods to meet the guidance of the journal.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also   indicate the acquisition and quantification methods for immunoblotting/western blots.

We double-checked and added catalogue numbers of all antibodies in the revision.

8) Microscope image acquisition: The following information must be   provided about the acquisition and processing of images:

a. Make and model of microscope

b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature

d. Imaging medium

e. Fluorochromes

f. Camera make and model

g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

We added the details of microscope image acquisition at the relevant places.

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

We double-checked the reference section.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

We mentioned at the end of the manuscript that

Online supplemental materials include Figure S1-S5 and proteomics data. Fig. S1 shows the supporting evidence about pVHL regulates SMAD3 stability in Hela cells and human patient tissues. Fig. S2 shows pVHL directly interact with SMAD3 for ubiquitination. Fig. S3 shows LxLxxP motif in the MH2 domain is indispensable for pVHL-mediated SMAD3 degradation. Fig. S4 shows pVHL impairs the activity of TGFß/SMAD3 signalling in cells. Fig. S5 shows dVHL negatively regulates TGFß/dSMAD signalling in *Drosophila* wing development.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

We added eTOC summary in the revision as follows

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

We added 'The authors declare no competing financial interests' in the manuscript.

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Yes, we did it

14) A separate author contribution section following the acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

We modified the author contribution in the revision.

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Sincerely,

Joan Massague
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the revised paper, Zhou and colleagues clarify many of the ambiguities generated to this reviewer. The revised paper presents a strong account on the ubiquitin ligase VHL, which can regulate TGFß signaling at the level of SMAD3. The combination of detailed biochemical assays with cell biological analysis convincingly show that pVHL is a SMAD3-interacting protein that ubiquitylates and degrades proteasomally SMAD3. This paper complements previous accounts on the mechanisms that regulate SMAD protein stability and also generates new interest regarding the potential co-regulation between oxygen sensing and TGF-beta signaling. I believe that this paper will make an important contribution to the multifaceted signaling by TGF-beta and to the prolific VHL field.

We would like to thank the reviewer for his/her final comments on the manuscript, and previous suggestions that helped to improve the quality of the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

Overall, the authors have satisfactorily addressed previously raised questions except for question #4.

In brief, my question aimed to explore if pVHL can mediate the degradation of SMAD2/3 in vivo. Based on the proposed working model, through ectopic expression of pVHL in the Drosophila, it is expected that less SMAD2/3 signal can be detected by immunostaining.

In the revised manuscript, the authors knocked down VHL, and then performed immunostaining on phosphorylated SMAD2/3. The staining results were inconclusive. The authors postulated that the antibody has cross-reactivity and might explain the negative results obtained.

Since the authors suggest that pVHL can mediate the degradation of total SMAD2/3regardless of phosphorylation status and successfully performed immunostaining on total SMAD2/3 in Fig.1E, it might be practical to conduct the immunostaining to explore if total SMAD2/3 are decreased upon dVHL overexpression in Drosophila.

We would like to thank the reviewer for the supportive and helpful comments. To address the remaining concern, we performed additional experiments by staining the Drosophila tissues with total Smad2 antibody from R&D systems (AF7948-SP). We confirmed that pVHL negatively regulates Smad protein levels in *Drosophila* wing imaginal disc (New Figure 5G-J).