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Ablation of Smurf2 reveals an inhibition in TGF-β signaling through mono-ubiquitination of Smad3

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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 11 April 2011

Thank you for submitting your manuscript on Smad3 monoubiquitination by Smurf2 for consideration by The EMBO Journal. We have now received the comments of three expert reviewers, copied below. While the referees consider your findings potentially interesting and the study overall well-done, they however raise serious concerns regarding the conclusiveness of the data and the experimental support for the main conclusions. The extent of this criticisms, I am afraid to say, appears to preclude publication in The EMBO Journal, at least in the present form. Nevertheless, given the interest in principle, I am willing to give you an opportunity to respond to the referees' criticisms through a revised version. It is however clear that for the manuscript to eventually become suitable for publication in these pages, a significant amount of revision effort will be required, and I also realize that not all of the requested manuscript may be technically feasible - I would therefore also understand if you were to decide to rather publish the manuscript rapidly and without major changes elsewhere. Should you however decide to thoroughly revise the study for The EMBO Journal, I would in fact not insist on clarification of the underlying genetic redundancies among Smad-regulating ubiquitin ligases, nor on the absence of mouse phenotypes. What would however be absolutely required is a thorough mechanistic clarification on the cellular level, in particular to show that Smad3 is regulated by Smurf2 on the endogenous level in the absence of exogenous overexpression. It will also be essential to address the question of mono- vs. multi-mono- vs. poly-ubiquitination raised by all referees, and to clarify the impact of Smurf2 on Smad2 relative to Smad3. Finally, the referees are unconvinced by the present evidence for Smad3 monoubiquitination affecting signaling via complex disruption, and more compelling data will be needed to address this.

Should you be confident that you may be able to address these major issues (as well as the various

more specific points) and to resubmit a revised version to our journal, I would encourage you to take the time to carefully address the criticisms. Although we normally allow only three months for a major revision, I would be willing to extend this to up to six months if needed in this case - competing manuscripts published elsewhere during this revision period would in this case not negatively affect our final decision on your manuscript (although you should rapidly inform the editors in such an instance, to discuss how to proceed). I do have to point out however that we allow only one round of major revision, and that we will therefore only be able to consider a revised version further if the referees felt their main issues had been largely satisfied at that point. Should you need any further clarification on anything related to this decision, please do not hesitate to contact me.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Novelty
- Smurf2 -/- MEF showed augmented TGFb response without affecting the steady state level or stability of main components of the signaling pathway.
- Smad3 is mono-ubiquitinated in MEF, and Smurf2 is responsible for this ubiquitinination.
- Ubiquitination of Smad3 depends on the phosphorylation of T179.
- Ubiquitination of Smad3 is on the multiple lysine of MH2 domain, results in reduced Smad4 binding capacity.
- In general, I think this is a good work and that deserves to be well considered for EMBOJ.

Weaknesses
- All the experiments are performed in MEF with overexpressed Smurf and it is not clear whether this regulation is general or specific in this cell type.
- KO mice are healthy and the biological effect of loss-of-Smurf2 is poor.
- Smad2 mono-ubiquitination is not affected by loss-of-Smurf2 (Fig. 4), whereas ARE-lux activity is increased (Fig. 2C).
- polyubiquitinatin by HECTs are not well-discussed.

Comment
This manuscript describes a novel mechanism of TGFb signal regulation, Smad3 mono-ubiquitination the by well-known E3 ligase Smurf2.
Authors generated a knockout of Smurf2, and the mice showed almost no developmental defect (Fig. 1). This is consistent with Smurf2-/- data by the another group. As Smurf2 is known regulator of TGFb signaling, authors examined the effect of loss of Smurf2 on TGFb responses. Using Smurf2-/- MEF, they found that both exogenous luciferase reporters and endogenous target gene expressions are augmented by loss of Smurf2 (Fig. 2). As Smurf2 has been reported to ubiquitinate and regulate the stability of TGFb type I receptor and Smad2/3, authors they next checked the stability of these proteins after TGFb stimulation, and found that it is not affected in Smurf2-/- MEF (Fig. 3). Authors then turned their attention to the role of Smurf2 on the ubiquitination of Smad. They found that in physiological conditions, Smad3 (and also Smad2) is mainly mono-ubiquitinated rather than poly-ubiquitinated, and that this ubiquitination depends on Smurf2 (Fig. 4). Authors further showed that Smurf2 directly ubiquitinate Smad3 and the binding of Smad3 and Smurf2 depends on PY motif and T179 phosphorylation of Smad3 protein (Fig. 5). Authors mapped the ubiquitinaiton site of Smad3 onto 4 lysines in MH2 domain (Fig. 6), and this suggested a molecular mechanism that the ubiquitination inhibits/disrupts the formation of Smad3/4 heteromeric complex

as well as Smad3 homomeric complex (Fig. 7). At last, loss-of-Smurf2 is shown to retard the nuclear export of Smad2/3 protein, supporting the idea that it affected Smad2/3/4 complex formation (Fig. 8).

In general the experiment is well conducted and the results are clearly shown.
There are several interesting findings;
1) Smad2/3 is mono-ubiquitinated in physiological condition,
2) Smurf2 is the responsive E3 ligase at least for Smad3,
3) this rather regulative - but not degradative modification is significant for negative regulation on TGFb signaling, and can contribute to the understanding of TGFb signaling pathway regulation.

That said, authors should clarify several uncertainties and strengthen their biological assays.

1) Authors should clarify the difference (or generality) between Smad2 and Smad3 more clearly. In Fig. 2 they show how ARE-lux is promoted by loss of Smurf2. this indicates the role of Smurf2 on Smad2 function. In Fig. 4, however, they did not show the effect of loss of Smurf2 on Smad2 ubiquitination and just show that add-back of Smurf2 slightly increased the ubiquitination level of Smad2. Nonetheless, authors used Smad2 and Smad3 as equivalent molecules in their biological assays as in Fig. 7a or Fig. 8.

2) The biochemical experiments are performed only in MEFs and upon overexpression of Smad2/3 protein. Please test if mono-ubiquitinaion of Smad2/3 occurs also in other cells, and if endogenous Smad3 in MEF is mono-ubiquitinated or not. This is important to exclude any artifact by the overexpression of tagged protein, and also because the other groups have shown they are polyubiquitinated in another cell line. (see Gao et al 2009 MolecularCell for Smad2, and Guo et al. 2008 Genes & Dev.22: 106-120 for Smad3)

3) It is not clear whether Smad3 ubiquitination by Smurf2 is constitutive, or serves as negative feedback/signalling termination. Finding that TGFb increases Smad3 ubiquitination (Fig. 4a) does indicate the latter possibility. Authors discussed Smurf2 as a R-smad counterpart of Ectodermin, both act as "disruptases". However, while Ectodermin is known to act in nucleus, the same is not clear for Smurf2. It is important to show when and where the ubiquitination is occurring; what Smad3 is it targeting? An interesting scenario, in line with the authors' hypothesis is that Smurf may operate in the nucleus.
Is it known where Smurf2 localizes with or without TGFb in MEF? is C-term phosphorylation of Smad3 affecting its ubiquitination?

4) related to the points above, authors show that Smurf2/ Smad3 binding depends on T179 phosphorylation. this phosphorylation is known to be induced by TGFb activation. Thus, it is possible that endogenous Smad3 is mono-ubiquitinated only upon TGFb signaling (or at least increase substantially). is this the case ?

5) Compared to abundant biochemical experiments, their biological/functional assessment of loss of Smurf2/gain-of-Smad3 monoubiquitination is limited. Authors should test some biological aspects of Smurf2-/- MEFs such as TGFb induced growth arrest or cell migration.

6) How does this regulative Smad3 mono-ubiquitination reconcile with previous publications on degradative poly-ubiquitination of the protein? Are these continuous regulations on TGFb signaling, or completely independent phenomena? Does polyubiquitination occur on the mono-ubiquitinated lysine? Is ACD or Q mutant poly-ubiquitinated under the condition where wildtype Smad3 is poly-ubiquitinated?

7) For the mapping of the lysine in FIg. 6, all the experiments are performed under the overexpression of Smurf2 protein, which may induce unphysiological ubiquitination. Also Fig. 6B shows Smurf2 CAN ubiquitinate FL or LC construct of Smad3, but this does not prove that these are physiological target (also, as it typically happens for domain deleted proteins, all are ubiquitinated; how can we know that only the ubiquitination of FL and LC are relevant?).
This section should be deleted or addressed in much more depth. I fear that this may require a dedicated study on its own.

8) Minor point (perhaps to be argued only in writings): I am convinced that the experiments of Figure 8 fully support the authors' conclusions. It is based on a speculation that the complex formation with Smad4 promotes nuclear retention of Smad2/3, which is very likely, but I do not believe that was ever formally shown. Schmierer et al. showed Smad2 D300H, which cannot bind Smad4, remains mainly in cytoplasm even after the TGFb signaling, but did not show that this depends on Smad4 binding. Thus, to make the point of the experiment, authors should show a) loss of Smad4 increase Smad2/3 nuclear export rate in wild type cell; and b) loss of Smad4 rescues the phenotype of Smurf2-/- cell (i.e. nuclear exporting rate is expected to be equal to Smad4 depleted wild type cell)

Referee #2 (Remarks to the Author):

Tang et al. reported potentially interesting findings that Smurf2, a HECT type E3 ligase, induces mono-ubiquitination of Smad3, and disrupts the oligomer formation of Smads. They generated Smurf2-null mice, and experiments were performed using Smurf2-null MEFs.

Critiques
1. Smurf2-null mice exhibited no overt developmental defect during embryogenesis. Thus, expression of other HECT type E3 ligases may be elevated and compensate for the lack of Smurf2. They should determine the expression levels of other HECT type E3 ligases in Smurf2-null MEFs, and show them as supplementary data.
2. Fig. 4. Although 72-kDa Smad3 bands were mainly detected by transfection of Smurf2, higher molecular bands were also detected in some figures, for example, in Fig. 4C, lanes 8 and 9. This suggests that Smurf2 induces both mono-and oligo-ubiquitination. They used only Ub(KO) to confirm mono-ubiquitination, but they should generate other ubiquitin mutants, and confirm whether Smurf2 indeed induces mono-ubiquitination of Smad3. Fig. 4D indicates that poly-ubiquitination of Smad2 was induced by Smurf2.
3. Fig. 7. Fig. 7D suggests that Smad4 binds to unmodified Smad3, but not to mono-uiquitinated Smad3. However, mono-ubiquitinated Smad2/3 are not visible in Fig. 7A and C, and they may be a very small fraction compared to unmodified Smad2/3. Are the amounts of mono-ubiquitinated Smad2/3 sufficient to explain the increase in the amounts of co-immunoprecipitated Smad4 in Smurf2-null MEFs? If the Smad complex was immunoprecipitated by Smad4 antibody, only unmodified Smad2/3 is co-immunoprecipitated in Fig. 7A.
4. It is not shown whether the induction of mono-ubiquitination of Smad3 is specific to Smurf2 or not. Smurf2, but not Smurf1, has been reported to interact with Smad2/3. Do Smurf1 and Nedd4L rescue the lack of Smurf2?

Referee #3 (Remarks to the Author):

Zhang and colleagues (and simultaneously Feng and colleagues) first cloned Smurf2 and immediately established the role of Smurf2 as an E3 ubiquitin ligase that leads to proteasomal degradation of Smad2 (Zhang et al. 2001 PNAS, Lin et al 2000 JBC). In their new paper Zhang and colleagues created a knockout mouse for Smurf2 and essentially find no phenotypes that link the function of Smurf2 to TGFbeta/Smad signaling. This confirms an independent report on a Smurf2 knockout that failed to demonstrate a TGF-beta family-dependent phenotype (Narimatsu et al. 2009 Cell). The new paper by Tang et al argues that these major discrepancies in the role of Smurf2 in TGF-beta signaling are due to the fact that all previous studies were based on overexpression of Smurf2 in various in vitro cell models. Thus, in order to rescue this problem of the field, they overexpress Smurf2 in cells derived from the knockout mice and cultured in vitro and report on a mono-ubiquitination of Smad3, another signal transducer of TGF-beta family members. Based on the logic of Tang et al presented in the introduction and first part of their paper, I would like to suggest that their findings on Smad3 are also an artifact of Myc-Smurf2 overexpression in a Smurf2-null genetic background. No evidence of Smad3 ubiquitination by Smurf2 at the endogenous physiological level is provided. In addition, I find the use of the term "mono-ubiquitination" not properly substantiated as the protein band that represents mono-ubiquitinated Smad3 corresponds to Smad3 with 2 ubiquitins attached to it. This is either a di-ubiquitination or a mono-ubiquitination at 2 distinct sites simultaneously that has been termed multi-ubiquitination in the ubiquitin field. I therefore recommend not using the term mono-ubiquitination unless this is rigorously proven. These

are my major concerns that make the central message and conclusions of this paper seriously doubtful. I provide below a long list of comments on the specific experiments presented in the Tang et al paper, but I cannot suggest any meaningful experiments that will explain a role of Smurf2 in TGF-beta signaling and thus improve this paper. I think Tang et al convincingly show that Smurf2 has no function in regulating TGF-beta signaling. Unless all of its function is rescued by Smurf1 that remains active in the cells. Then this should be shown instead.

Specific major points:
1. The knockout mouse produced allows the expression of the N-terminal domain of Smurf2 that encompasses its C2 and first WW domains. No information on this is provided in the paper. Is this N-terminal domain expressed in the mice? What are the functional consequences of expression of such a mutant Smurf2 protein?
2. The differences in TGF-beta gene and promoter responses shown in Figure 2 between WT and KO cells are essentially negligible. The promoter luciferase experiments show that Smurf2 KO enhances the basal levels of promoter expression. The TGF-beta-inducible levels of the promoters are essentially much smaller than in WT cells. This is the case for all promoters. This evidence is not compatible with a negative role of Smurf2 in TGF-beta signaling. The time course experiments of endogenous gene expression of Figure 2 also demonstrate small quantitative effects on some genes and in specific time points. No difference is seen in very well-established Smad3-regulated gene such as c-Jun and Smad7 during the first 4 hours of TGF-beta signaling. How is this compatible with the molecular model of Smad3 ubiquitination shown in the rest of the paper? The data of figure 2 show convincingly that the effects of Smurf2 on TGF-beta-regulated gene expression must be due to other molecular targets of Smurf2 and not Smad complexes as the authors propose. In the discussion, page 18, the authors suggest that Smurf2 is a physiological inhibitor of a subset of TGF-beta-induced transcriptional responses. Why is this so? Which is the subset of genes? Are these genes regulated by Smad3 or by other signaling mediators downstream of TGF-beta receptors? One possibility for the lack of effects of the Smurf2 KO is the compensation by Smurf1. This is not discussed sufficiently in this paper. Is Smurf1 expressed in the Smurf2 KO cells? What happens to the TGF-beta gene responses and to the stability of Smads when siRNA against Smurf1 is transfected in the Smurf2 KO cells? Do now the cells show dramatic differences compared to WT and plain Smurf2 KO cells?

3. The data of figure 3 are clear but compare 2 independent cell lines, so they may reflect cell line clonal variations. They need to be repeated in multiple independent WT and KO cell lines for statistical reasons and in addition, they should be compared to the rescue condition of KO cells plus Smurf2 overexpression as it is done in the rest of the paper.

4. Smad3 mono-ubiquitination: The paper consistently describes the mono-ubiqutinated Smad3 band of 77 kDa when the core Smad3 band is 55 kD, thus leading to a ubiquitin size of 17 kDa that almost perfectly fits to 2 ubiquitins. This cannot be a mono-ubiquitination of a single lysine. It is either a stable intermediate of a poly-ubiquitination as shown in many of the blots with the 2 ubiquitins attached on a single lysine acceptor or mono-ubiquitination on 2 separate lysines, a phenomenon that has been classified as multi-ubiquitination. The latter is supported by the authors and is depicted also in the cartoon of the last figure. Based on this, it is better if the term multi-ubiquitination replaces the term mono-ubiquitination throughout the paper. The paper implies that Smad3 ubiquitination is the real in vivo role of Smurf2 that explains how it regulates TGF-beta signaling (which it does not, see above). However, all ubiquitination experiments are performed with overexpressed myc-tagged Smurf2. Thus I cannot understand how this differs from previous reports and furthermore we do not see data on the stability of Smads after myc-Smurf2 transfection. For the major conclusion of this paper to stand the authors need to demonstrate ubiquitination of endogenous Smad3 with endogenous Smurf2 in WT cells. What is the pattern of ubiquitination of Smad3 in such cells? Smad3 also appears to bind promiscuously ubiquitin. Thus the classical IP-western blots used in this study are not sufficient evidence. An independent means of showing the ubiquitination, e.g. via mass-spec analysis is required. The Smad3(Ub) used in figure 7D is an ideal protein to perform such mass spec studies although it is produced in vitro, so it may not reflect what happens in vivo.

5. Smad3 ubiquitination sites: Figure 6B shows that both NL and LC domains of Smad3 become rather well ubiquitinated. This happens even in the absence of transfected Smurf2, suggesting the role of Smurf1 or other Ub ligases that probably are expressed in the cells. From this figure it

appears as the strongest oligo-ubiquitination of Smad3 affects the NL domain, while the LC domain shows a beautiful ladder of ubiquitinated bands plus a smear all the way to the top of the gel. Why are these ladders and smears called mono-ubiquitination when they are classically called poly-ubiquitination? Via which lysine in the ubiquitin protein (K48, K63, K27 etc) are these ladders and smears built? The experiment with Ub(K0) is not relevant (Figure 4B). Using this ubiquitin mutant, all ubiquitinated proteins become only mono-ubiquitinated, as this ubiquitin cannot support the building of ladders. Thus, I think that mapping the lysines of the NL domain is equally important as mapping those in the LC domain. A corollary of this is that the effect of Smad3 ubiquitination on its function may involve both NL and LC domains, and thus may not be the one proposed by this study.

6. Smad3-Smad4 complexes and the role of ubiquitination (disruptase): some of the experiments using endogenous Smads are misleading. The Smad2/3 antibody is shown to precipitate lots of Smad2 but then the authors conclude that Smurf2 affects the complex of Smad3 with Smad4. Does this Smad2/3 antibody precipitate any Smad3? I think this antibody is not appropriate. Endogenous Smad3 should be precipitated with a Smad3-specific antibody, which the authors use in other figures. Figure 7B and the results importantly show that the lysine mutants (ACD and Q) cannot be phosphorylated by the receptor and thus cannot form oligomers with Smad4. How then can one use these mutants to prove that Smad3 ubiquitination in these lysines affects Smad3-Smad4 oligomerization? The ACD and Q mutations probably destroy the folding of the MH2 domain and these mutant Smad3 proteins cannot function at all. Thus the ubiquitination sites have not been proven yet and mutagenesis is not the right approach. Mass spec is better. The authors propose that Smurf2 ubiquitinates Smad3 in the complex with Smad4 and thus acts as a disruptase. No evidence for this is shown. Are the 4 lysines of the MH2 domain available for ubiquitination in the oligomeric complex? The authors imply that they are not. Then, how do they become ubiquitinated by Smurf2? Does Smurf2 bind to Smad3 when it is in complex with Smad4? I get the idea that if the model has any relevance to the in vivo situation, Smad3 gets ubiquitinated and this prohibits oligomerization as the cartoon shows. Thus, why use the misleading term "disruptase" and discuss such a model?

7. Smad nuclear shuttling and the role of Smurf2: Figure 8 uses again the Smad2/3 antibody that recognizes mainly Smad2. Yet the interperatation of these experiments is centering around Smad3! Anti-Smad3-specific antibodies need to be used for the immunofluorescence experiments. The same for figure 8B. The case is made for Smad3, yet the controls are measuring pSmad2 and Smad2. Why? pSmad3 and Smad3 should be measured. The results in page 22 top section claim that no effects on C-terminal serine phosphorylation of endogenous Smads were measured, but the figure shows only Smad2.

Specific minor/technical comments:
1. Figure 4: many blots are over-saturated (including the long exposures but also some of the regular exposures) and this precludes rigorous evaluation of expression levels. Less saturated exposures would be better. Figure 4B lacks the control of Flag-Smad3 without Myc-Smurf2 co-transfection. Figure 4E second panel (WCL/Flag) should explain which of the multiple protein bands are specific and which are not. Figure 4F should show longer exposure of the right hand side Smad3 blot and it will probably reveal the ladder of poly-/multi-ubiquitinated Smad3 as the corresponding HA(Ub) blot on the left shows.
2. Figure 5: panel A is saturated and it is difficult to see which are the specific bands and which are background shadows. Better contrast is needed. In panel D it is difficult to see the extra protein bands on top of the overexposed Smad3 band (Flag IP long exposure). An SDS-PAGE of better resolution is somehow needed.
3. Figure 6C: a lower exposure and an ubiquitin blot are needed to obtain convincing evidence about the role of the specific mutations on Smad3 ubiquitination.
4. Figure 7A (Smad2 blot), 7B (Smad3 blot in Flag IP and Flag blot in WCL), 7C (both top and bottom blots) are saturated. Lower exposures are needed for accurate evaluation of differences in protein levels.
5. Results, page 9, start of the second paragraph: The authors describe that "it is generally believed based on previous biochemical and cell culture studies that Smurf2 regulates TGF- signaling by targeting Smads or the type I receptor for proteasomal degradation". A review is cited for this. It will be more honest to the non-specialist reader of the paper if the term "believed" is removed. In science beliefs should not be of importance. Better to replace the word believed with the word "demonstrated" or "established". The 3 key primary references should be cited here instead of a review article: Zhang et al. 2001, Lin et al. 2000, Kavsak et al. 2000.

6. Discussion, page 20 middle: minor typo in the sentence: A possible explanation for this discrepancy may is that the previous study...

1st Revision - authors' response 31 August 2011

Responses to reviewers:

We thank the reviewers for their effort in evaluating our manuscript and for their constructive comments. We have augmented our observations with additional experiments as demanded and made appropriate modifications in the manuscript accordingly. We believe these amendments have strengthened our conclusions. Here are our responses to each individual comment.

Reviewer 1:

1) Authors should clarify the difference (or generality) between Smad2 and Smad3 more clearly. In Fig. 2 they show how ARE-lux is promoted by loss of Smurf2. this indicates the role of Smurf2 on Smad2 function. In Fig. 4, however, they did not show the effect of loss of Smurf2 on Smad2 ubiquitination and just show that add-back of Smurf2 slightly increased the ubiquitination level of Smad2. Nonetheless, authors used Smad2 and Smad3 as equivalent molecules in their biological assays as in Fig. 7a or Fig. 8.

Apparently there are 2 different versions of ARE-Luc reporter in the TGF- field as well as in our lab. The 3XARE-Luc reporter (aka: A3-luc) that we used in the previous version of this manuscript does respond to Smad3 as shown by others (Liu et al., MCB, 1999, 19, 424-430). We have tested a different version of ARE-Luc: ARE-Lux (aka: 3AR-Luc), which does not respond to Smad3. Both ARE reporter constructs contain three copies of the activin response element from the Mix2 promoter, and are originated from the same lab but constructed by two different persons. We do not know what makes these two different ARE-Luc reporters different, but clearly one is responsive to Smad3, whereas the other is not. Using the latter ARE-Lux, we now show that loss of Smurf2 only causes a slight increase in the Smad2-mediated transcriptional output (Figure 2C). We also repeated the experiments in the revised Figure 7A and Figure 8 using a Smad3-specific antibody and obtained similar results, but which are more specific to Smad3.

2) The biochemical experiments are performed only in MEFs and upon overexpression of Smad2/3 protein. Please test if mono-ubiquitinaion of Smad2/3 occurs also in other cells, and if endogenous Smad3 in MEF is mono-ubiquitinated or not. This is important to exclude any artifact by the overexpression of tagged protein, and also because the other groups have shown they are polyubiquitinated in another cell line. (see Gao et al 2009 MolecularCell for Smad2, and Guo et al. 2008 Genes & Dev.22: 106-120 for Smad3)

We have now demonstrated that Smurf2-induced mono-ubiquitination of endogenous Smad3 also occurs in MEFs (Figure 4G) and Hela cells (data not shown). Moreover, transfected Flag-Smad3 in HEK293 cells is also subject to mono-ubiquitination (Figure 7D).

3) It is not clear whether Smad3 ubiquitination by Smurf2 is constitutive, or serves as negative feedback/signalling termination. Finding that TGFb increases Smad3 ubiquitination (Fig. 4a) does indicate the latter possibility. Authors discussed Smurf2 as a R-smad counterpart of Ectodermin, both act as "disruptases". However, while Ectodermin is known to act in nucleus, the same is not clear for Smurf2. It is important to show when and where the ubiquitination is occurring; what Smad3 is it targeting? An interesting scenario, in line with the authors' hypothesis is that Smurf may operate in the nucleus.
Is it known where Smurf2 localizes with or without TGFb in MEF? is C-term phosphorylation of Smad3 affecting its ubiquitination?

Smurf2 is located both in the nucleus and the cytoplasm with or without TGF- stimulation in MEFs (Supplemental Fig. S5); C-term phosphorylation of Smad3 does not affect its ubiquitination (Figure 5E). Therefore, we step back from implying that Smurf2 acts as a "disruptase" in the nucleus, and modify the text accordingly. But clearly, ubiquitination of Smad3 blocks Smad complex formation,

and TGF- slightly increases ubiquitination of both transfected FLAG-Smad3 and endogenous Smad3 (Figure 4A and Figure 4G). Since this regulation requires phosphorylation of T179 in the Smad3 linker, it constitutes a negative feedback regulation.

4) related to the points above, authors show that Smurf2/ Smad3 binding depends on T179 phosphorylation. this phosphorylation is known to be induced by TGFb activation. Thus, it is possible that endogenous Smad3 is mono-ubiquitinated only upon TGFb signaling (or at least increase substantially). is this the case ?

Yes, our data presented in Figure 4A is consistent with the idea of mono-ubiquitination of Smad3 is enhanced by TGF- stimulation, which induces phosphorylation of T179. However, since the basal level of T179 phosphorylation is quite high in cells, it is difficult, if not impossible, to definitely demonstrate this point experimentally. Nevertheless, our data show that TGF- treatment could further increase Smad3 ubiquitination, which likely constitutes a negative feedback regulation for attenuating signaling. With regard to demonstration of mono-ubiquitination of endogenous Smad3, we add Figure 4G to show this point, and further show that the levels of mono-ubiquitined endogenous Smad3 in wild type MEFs is substantially increased upon TGF- treatment.

5) Compared to abundant biochemical experiments, their biological/functional assessment of loss of Smurf2/gain-of-Smad3 monoubiquitination is limited. Authors should test some biological aspects of Smurf2-/- MEFs such as TGFb induced growth arrest or cell migration.

We have now included results showing that loss of Smurf2 renders primary MEFs more sensitive to TGF- -mediated growth inhibition (Figure 2H).

6) How does this regulative Smad3 mono-ubiquitination reconcile with previous publications on degradative poly-ubiquitination of the protein? Are these continuous regulations on TGFb signaling, or completely independent phenomena? Does polyubiquitination occur on the mono-ubiquitinated lysine? Is ACD or Q mutant poly-ubiquitinated under the condition where wildtype Smad3 is poly-ubiquitinated?

Previously, Xiao-Fan Wangís group (Gao et al 2008, Genes & Dev) showed that in the absence of TGF- stimulation, GSK3 phosphorylates Smad3 and causes Smad3 polyubiquitinated and targeted for degradation. Under their experimental conditions, we found that the Smad3 Q mutant could be poly-ubiquitinated just as the wild type Smad3 (Supplemental Fig. S6), suggesting these two types of ubiquitin modification are likely to be separate event since most proteins eventually turnover via the proteasome pathway. Therefore, it is possible that Smurf2 induces mono-ubiquitination through phosphorylation control at T179, and GSK3 induces poly-ubiquitinatition through phosphorylation at N-terminal T66. But it is not known if these two types of regulation are inter-convertible under any other conditions.

7) For the mapping of the lysine in FIg. 6, all the experiments are performed under the overexpression of Smurf2 protein, which may induce unphysiological ubiquitination. Also Fig. 6B shows Smurf2 CAN ubiquitinate FL or LC construct of Smad3, but this does not prove that these are physiological target (also, as it typically happens for domain deleted proteins, all are ubiquitinated; how can we know that only the ubiquitination of FL and LC are relevant?).
This section should be deleted or addressed in much more depth. I fear that this may require a dedicated study on its own.

We agree with this reviewer that overexpression of protein fragments often leads to non-physiological ubiquitination. Moreover, such protein fragments tend to form aggregates with other ubiquitinated proteins in the proteasome, appearing as artificially ubiquitinated on anti-HA-Ub Western blots. To circumvent this problem, we replaced anti-HA(Ub) blot with anti-Flag-Smad3 blot in the revised Figure 6B, which shows that only Smad3 FL and LC fragments were ubiquitinated in the presence of Smurf2. This new experiment ruled out the possibility of detecting associated ubiquitin modified proteins and showed that Smad3 ubiquitination is dependent upon the presence of the linker region that is required for Smurf2 binding. Taken these precautions into account, we believe that the data presented in the new Figure 6B, given the caveat mentioned above, are relevant and indeed reflect ubiquitination of the full Smad3.

8) Minor point (perhaps to be argued only in writings): I am convinced that the experiments of Figure 8 fully support the authors' conclusions. It is based on a speculation that the complex formation with Smad4 promotes nuclear retention of Smad2/3, which is very likely, but I do not believe that was ever formally shown. Schmierer et al. showed Smad2 D300H, which cannot bind Smad4, remains mainly in cytoplasm even after the TGFb signaling, but did not show that this depends on Smad4 binding. Thus, to make the point of the experiment, authors should show a) loss of Smad4 increase Smad2/3 nuclear export rate in wild type cell; and b) loss of Smad4 rescues the phenotype of Smurf2-/- cell (i.e. nuclear exporting rate is expected to be equal to Smad4 depleted wild type cell)

This is an intriguing proposition since both Caroline Hill and Xuedong Liuís groups have shown using quantitative biology approaches that formation of Smad complexes increased nuclear accumulation of Smad2/3. However, we feel that to rigorously address if loss of Smad4 increases the nuclear export rate of Smad2/3 requires considerable experimental undertaking and is beyond the scope of this current manuscript.

Reviewer 2:

1. Smurf2-null mice exhibited no overt developmental defect during embryogenesis. Thus, expression of other HECT type E3 ligases may be elevated and compensate for the lack of Smurf2. They should determine the expression levels of other HECT type E3 ligases in Smurf2-null MEFs, and show them as supplementary data.

While we previously demonstrated a compensatory increase of Smurf2 expression in Smurf1-/- MEFs, no such change can be said about Smurf1 in Smurf2-/- cells (Supplemental Fig.S1B). Nor did we find any increase in the expression of several other HECT-domain E3 ligases (Supplemental Fig. S1B). Nevertheless, we included these data according to this reviewerís request.

2. Fig. 4. Although 72-kDa Smad3 bands were mainly detected by transfection of Smurf2, higher molecular bands were also detected in some figures, for example, in Fig. 4C, lanes 8 and 9. This suggests that Smurf2 induces both mono-and oligo-ubiquitination. They used only Ub(KO) to confirm mono-ubiquitination, but they should generate other ubiquitin mutants, and confirm whether Smurf2 indeed induces mono-ubiquitination of Smad3. Fig. 4D indicates that poly-ubiquitination of Smad2 was induced by Smurf2.

In our hands, we only detected high molecular weight (presumably poly- or oligo-unibquitinated) Smad3 when it was co-expressed with HA-Ub and by anti-HA Western. This approach artificially favors the poly-ubiquitinated proteins due to the selective enrichment of ubiquitin in such protein species. However, when we examined the level of ubiquitinated protein products with antibodies against Smad3 itself, we could only detect the 72 kD, mono-ubiquitinated Smad3 plus one or two additional steps (should correspond to di- or tri-ubiquitination), but never poly-ubiquitinated Smad3 as with anti-HA-Ub. As we argued in the revised manuscript, although oligo-ubiquitinated Smad3 does form in cells, the ubiquitin chain is never allowed to extend to sufficient length to mark Smad3 for proteasomal degradation. Our experiment with Ub(KO) clearly demonstrated that the 72-KDa Smad3 is the result of attaching two ubiquitin moieties to two lysines, and now we further augmented this observation with Ub(K48R) and Ub(K63R) mutants, which generated similar results. In contrast, we observed oligo- and poly-ubiquitinated Smad2 that persisted even in the absence of Smurf2 (Fig.4B). So, again as we argued in the manuscript, these two Smads behave differently in the ubiquitination reaction.

3. Fig. 7. Fig. 7D suggests that Smad4 binds to unmodified Smad3, but not to mono-uiquitinated Smad3. However, mono-ubiquitinated Smad2/3 are not visible in Fig. 7A and C, and they may be a very small fraction compared to unmodified Smad2/3. Are the amounts of mono-ubiquitinated Smad2/3 sufficient to explain the increase in the amounts of co-immunoprecipitated Smad4 in Smurf2-null MEFs? If the Smad complex was immunoprecipitated by Smad4 antibody, only unmodified Smad2/3 is co-immunoprecipitated in Fig. 7A.

This is a very interesting issue since indeed we had to prolong the exposure time in order to visualize the mono-ubiquitinated form of Smad3 on anti-Flag-Smad3 blot. However, there is

essentially nothing known about the dynamic regulation of the mono-ubiquitinated Smad3 level in cells. For instance, we do not know if mono-Ub-Smad3 is actively turned over by a deubiquitin enzyme. Until and unless we are able to specifically block the Smad3 DUB, it would not be feasible to stoichiometrically reconcile the gains in the mono-ubiquitinated Smad3 by the loss of Smad3 complex formation. On a more technical note, according to Xuedong Liuís calculation (Clarke et al., IEE Proc-Syst Biol 2006, 153, 412-424), Smad3 is present in a much lower level than Smad4 in cells. Therefore, we are expected to detect more easily the difference in Smad3-bound Smad4 if we IP for Smad3 than the difference in Smad4-bound Smad3 if we IP for Smad4. Nevertheless, in GST-pull down assays described in the Figure 7D, we clearly demonstrated the preference of Smad3 and Smad4 for the unmodified Smad3 in homo- and heterotrimeric complex formation, respectively.

4. It is not shown whether the induction of mono-ubiquitination of Smad3 is specific to Smurf2 or not. Smurf2, but not Smurf1, has been reported to interact with Smad2/3. Do Smurf1 and Nedd4L rescue the lack of Smurf2?

We now include data in the Supplemental Fig. S3A to show that the ubiquitination pattern of Smad3 does not change between WT and Smurf1-/- MEFs, implying that Smurf1 does not regulate Smad3 ubiquitination under physiological condition. However, when overexpressed, Smurf1 does possess the ability to ubiquitinate Smad3 in Smurf2-/- cells (Supplemental Fig. S3B). Although Smurf1 was shown not to interact with Smad2/3 initially, this interaction was demonstrated subsequently by in vitro binding assays although at much lower affinity (Barrios-Rodiles et al., Science, 2005, 307, 1621-1625; Gao et al., Mol Cell, 2009, 36, 457-468). This is precisely the kind of over-expression artifacts that we try to avoid with our knockout analyses.

Reviewer 3:

This reviewer took issue with our approach of assaying for the function of Smurf2 by re-introducing it back into Smurf2-/- cells and argues that our "findings on Smad3 are also an artifact of Myc-Smurf2 overexpression in a Smurf2-null genetic background". We respectfully disagree with this assessment. First of all, the observation of Smad3 mono-ubiquitination was initially made in wild type MEFs with Flag-Smad3 and HA-Ub (Figure 4A). So, this modification was afforded onto Smad3 by an endogenous E3 ligase at the physiological level and it was absent in Smurf2-/- MEFs, which allowed us in part to attribute it specifically to Smurf2. We could not reach this conclusion with confidence without resorting to the lengthy genetic manipulation to inactivate the Smurf2 allele, precisely for the reasons that we have argued in the manuscript. Second, re-introducing Smurf2 back into Smurf2-/- MEFs allows us to assay for its function in a clean genetic background and obtain results that would not be attainable otherwise. Short of creating actual genomic mutations by "knock-in" approaches, this represents the best way to study structure-function relationship in a mammalian system. For instance, we observed that the E3 ligase activity of Smurf2 is absolutely required for Smad3 mono-ubiquitination since Myc-Smurf2CG is completely incapable of modifying Smad3 in Smurf2-/- MEFs, whereas Myc-Smurf2 is fully capable of. In contrast, Smad2 still exhibited a basal level of ubiquitin modification in Smurf2-/-MEFs expressing Myc-Smurf2CG. Again, without using the knockout genetic approach, one could never be certain that Smurf2 has a specific function in ubiquitinating Smad3. Third, many of our subsequent conclusions were reached based on contrasting difference between wild type and Smurf2-/- MEFs, thus carry specific physiological significance. Fourth our functional assessment of TGF- signaling as well as biochemical analysis of the stability of various pathway components were all carried out in knockout cells strictly, without over-expression of any transfected protein, thus are entirely physiological. Last but not the least, we now show in the revised Figure 4G that endogenous Smad3 also undergoes multi mono-ubiquitination, again validating our previous observation being physiological. We would also like to clarify that in Zhang et al (PNAS 2001), overexpression of Smurf2 was actually shown NOT to change Smad3 stability while it caused only a slight decrease of the Smad2 protein level. With regard to naming mono- versus multi- or di-ubiquitination in the original manuscript, we never meant to equate mono-ubiquitination to attachment of a single lysine residue of Smad3 molecule. In order to make ourselves more explicit, we modified the text substantially in the revised manuscript and now call this multiple monoubiquitination to preserve continuity with the part of published literature linking this type of ubiquitin modification with regulating of intracellular trafficking because the net effect that we observed on Smad3 ubiquitination is the change in the nuclear and cytoplasmic distribution of Smad3. Finally, we

believe that this reviewer completely misconstrued our results and conclusion in the general comments by stating that "Tang et al convincingly show that Smurf2 has no function in regulating TGF-beta signaling". Our data clearly show a function of Smurf2, or the lack of which in Smurf2-/- MEFs, in dampening the transcriptional and cell proliferative responses of TGF-beta (revised Figure 2).

We understand that this reviewer may have developed a prejudice against our study because of conflicting reports from different laboratories in the literature over Smurf functions in TGF- signaling. This is why we undertook lengthy genetic manipulation to create knockout mice to address Smurf functions under physiological conditions and this study represents the best approach that the current molecular and cellular biological technology allow to take. We hope that with the additional supporting evidence and the improved description in the revised manuscript, this reviewer can reach the same conclusion as we did with due objectivity and open-mindedness.

1. The knockout mouse produced allows the expression of the N-terminal domain of Smurf2 that encompasses its C2 and first WW domains. No information on this is provided in the paper. Is this N-terminal domain expressed in the mice? What are the functional consequences of expression of such a mutant Smurf2 protein?

As we showed previously, deleting the bulk of Smurf2 after the first WW domain is sufficient in ablating its function. To demonstrate this point and to satisfy this reviewerís concern, we performed real-time PCR experiment to show that the mutant Smurf2 transcript is expressed at a reduced level. We also constructed the residual Smurf2 fragment corresponding to the remaining of the mutant allele, and found that it is unable to interact with Smad3 or inhibit the transcriptional response of TGF- (Supplemental Fig. S1).

2. The differences in TGF-beta gene and promoter responses shown in Figure 2 between WT and KO cells are essentially negligible. The promoter luciferase experiments show that Smurf2 KO enhances the basal levels of promoter expression. The TGF-beta-inducible levels of the promoters are essentially much smaller than in WT cells. This is the case for all promoters. This evidence is not compatible with a negative role of Smurf2 in TGF-beta signaling. The time course experiments of endogenous gene expression of Figure 2 also demonstrate small quantitative effects on some genes and in specific time points. No difference is seen in very well-established Smad3-regulated gene such as c-Jun and Smad7 during the first 4 hours of TGF-beta signaling. How is this compatible with the molecular model of Smad3 ubiquitination shown in the rest of the paper? The data of figure 2 show convincingly that the effects of Smurf2 on TGF-beta-regulated gene expression must be due to other molecular targets of Smurf2 and not Smad complexes as the authors propose. In the discussion, page 18, the authors suggest that Smurf2 is a physiological inhibitor of a subset of TGF-beta-induced transcriptional responses. Why is this so? Which is the subset of genes? Are these genes regulated by Smad3 or by other signaling mediators downstream of TGF-beta receptors? One possibility for the lack of effects of the Smurf2 KO is the compensation by Smurf1. This is not discussed sufficiently in this paper. Is Smurf1 expressed in the Smurf2 KO cells? What happens to the TGF-beta gene responses and to the stability of Smads when siRNA against Smurf1 is transfected in the Smurf2 KO cells? Do now the cells show dramatic differences compared to WT and plain Smurf2 KO cells?

In primary MEF cultures, there is autocrine release of TGF- , which causes the luciferase reporter readout to be detected at a higher basal level. To rectify this situation, we added T RI inhibitor SB431542 to un-stimulated samples in the revised Figure 2A-C to reveal the true amplitude of TGF- signaling response. Nevertheless, the absolute level of TGF- stimulated transcriptional readout still increased dramatically in Smurf2-/- MEFs compared to the wild type control MEFs.

Indeed, the difference in c-Jun and Smad7 transcription between wild type and Smurf2-/- MEFs is most pronounced at 8 hours of TGF- treatment, as this reviewer pointed out. Considering that c-Jun and Smad7 are early response genes whereas PAI-1, CTGF as well as growth inhibition are long term responses (Zi et al., Molecular Systems Biology, 2011, 7, 492), the shape of their kinetic expression curves in Smurf2-/- MEFs is influenced by the activation as the result of Smurf2 loss and other factors including the natural decay of their messages. In any event the difference between the wild type and Smurf2-/- MEFs is significant (Figure 2D-G).

Smad2 and Smad3 both mediate common and specific transcriptional responses of TGF- . Because loss of Smurf2 only has a slight effect on Smad2 ubiquitination, its effect on Smad2-dependent transcriptional responses could be also small. We have demonstrated this point using a Smad2-specific version of ARE-Lux reporter (Figure 2C). Thus, as implied in the text, the subset of transcriptional responses affected by the loss of Smurf2 refers to those that are mediated specifically by Smad3. We have made this a more explicit point in the revised discussion.

Certainly, Smurf1 and Smurf2 share some redundant functions because the double KO mice are embryonic lethal, and we first reported the compensatory up-regulation of Smurf2 in Smurf1 KO mice (Yamashita et al, Cell 2005). However, unlike what is reported here in Smurf2-/- MEFS, Smad3-dependent transcriptional responses and Smad3 ubiquitination (Supplemental Fig. S3) did not change in Smurf1-/- MEFs. This indicates that at the physiological level, Smurf1 does not regulate Smad3 ubiquitination. Even in the embryonic extracts prepared from Smurf1 and Smurf2 double KO mice, we did not observe any change in Smad2/3 protein level (Supplemental Fig. S3), and Jeff Wranaís group also showed a lack of change in the p-Smad level in Smurf1/Smurf2 double KO embryonic tissues. So, we are certain that neither Smurf1 nor Smurf2 regulate Smad2/3 stability.

3. The data of figure 3 are clear but compare 2 independent cell lines, so they may reflect cell line clonal variations. They need to be repeated in multiple independent WT and KO cell lines for statistical reasons and in addition, they should be compared to the rescue condition of KO cells plus Smurf2 overexpression as it is done in the rest of the paper.

We are perplexed by this comment. It seems that this reviewer either did not read our manuscript carefully or did not really understand it. In this study, we isolated primary MEFs as pools of cells derived either from a single mouse E14.5 embryo or from a pool of embryos, but never established any clonal line of MEF cells for the manuscript proper. Nevertheless, we repeated the experiments described in Figure 3A using different POOL of MEFs to arrive at statistical assessment of the perceived variation and present the data in Figure 3B. The original set of data was shown as representative of the two.

To satisfy this demand, we stably re-introduced Smurf2 into an established/immortalized line of Smurf2-/- MEFs, and confirmed that restoring Smurf2 expression does not alter either p-Smad3 or total Smad3 protein levels. This part of the data is now added as Supplemental Fig. S2.

4. Smad3 mono-ubiquitination: The paper consistently describes the mono-ubiqutinated Smad3 band of 77 kDa when the core Smad3 band is 55 kD, thus leading to a ubiquitin size of 17 kDa that almost perfectly fits to 2 ubiquitins. This cannot be a mono-ubiquitination of a single lysine. It is either a stable intermediate of a poly-ubiquitination as shown in many of the blots with the 2 ubiquitins attached on a single lysine acceptor or mono-ubiquitination on 2 separate lysines, a phenomenon that has been classified as multi-ubiquitination. The latter is supported by the authors and is depicted also in the cartoon of the last figure. Based on this, it is better if the term multi-ubiquitination replaces the term mono-ubiquitination throughout the paper. The paper implies that Smad3 ubiquitination is the real in vivo role of Smurf2 that explains how it regulates TGF-beta signaling (which it does not, see above). However, all ubiquitination experiments are performed with overexpressed myc-tagged Smurf2. Thus I cannot understand how this differs from previous reports and furthermore we do not see data on the stability of Smads after myc-Smurf2 transfection. For the major conclusion of this paper to stand the authors need to demonstrate ubiquitination of endogenous Smad3 with endogenous Smurf2 in WT cells. What is the pattern of ubiquitination of Smad3 in such cells? Smad3 also appears to bind promiscuously ubiquitin. Thus the classical IP-western blots used in this study are not sufficient evidence. An independent means of showing the ubiquitination, e.g. via mass-spec analysis is required. The Smad3(Ub) used in figure 7D is an ideal protein to perform such mass spec studies although it is produced in vitro, so it may not reflect what happens in vivo.

Once again, this reviewer completely missed our point. As in the rebuttal to his/her general concerns, we stated explicitly throughout the manuscript that the 72KD Smad3 protein represents attachment of two ubiquitin moieties on two separate lysine residues, which is, by the established and well-published terminology, defined as mono-ubiquitination. We never stated, explicitly or implicitly, that mono-ubiquitination represents addition of a single ubiquitin to one lysine residue of

the modified protein. See the above rebuttal to major concerns for a full response to this critique.

We are well aware of the fact that ubiquitin tend to bind proteins such as Smad3 promiscuously, thus purposely analyzed Smad3 itself in many IP-Western experiments to exclude non-specific proteins that may appear as those higher MW bands in anti-HA-Ub blot. Indeed, by this approach we exposed the mono-ubiquitin nature of Smurf2-mediated modification of Smad3.

We agree with this reviewer that mass spec analysis in theory would be a valid alternative method for identifying ubiquitin attachment sites. We have tried more than 12 mass spec experiments with purified ubiquitinated Smad3 at a NCI core facility, but were never able to recover any peptide from the MH2 domain of Smad3 after trypsin or chemotrypsin digestion. Unfortunately, no useful information was yielded by this approach.

5. Smad3 ubiquitination sites: Figure 6B shows that both NL and LC domains of Smad3 become rather well ubiquitinated. This happens even in the absence of transfected Smurf2, suggesting the role of Smurf1 or other Ub ligases that probably are expressed in the cells. From this figure it appears as the strongest oligo-ubiquitination of Smad3 affects the NL domain, while the LC domain shows a beautiful ladder of ubiquitinated bands plus a smear all the way to the top of the gel. Why are these ladders and smears called mono-ubiquitination when they are classically called poly-ubiquitination? Via which lysine in the ubiquitin protein (K48, K63, K27 etc) are these ladders and smears built? The experiment with Ub(K0) is not relevant (Figure 4B). Using this ubiquitin mutant, all ubiquitinated proteins become only mono-ubiquitinated, as this ubiquitin cannot support the building of ladders. Thus, I think that mapping the lysines of the NL domain is equally important as mapping those in the LC domain. A corollary of this is that the effect of Smad3 ubiquitination on its function may involve both NL and LC domains, and thus may not be the one proposed by this study.

As reviewer 1 points out, truncated proteins tend to become heavily ubiquitinated because they are prone to be cleansed from the cell. This reviewer also made a valid point that ubiquitin tends to bind other proteins promiscuously. So, IP FLAG-Smad3 and blot Ub is never a good approach to visualize authentic ubiquitination of Smad3 proper. Because of this concern, we replaced the original anti-HA-Ub blot with anti-FLAG-Smad3 blot following immunoprecipitation for Smad3 in the revised Figure 6B to ascertain that those higher molecular weight bands actually represent modified Smad3 rather than proteins associated with ubiquitin. As shown in the top panel of revised Fig. 6B, Smurf2 induced ubiquitination on full-length Smad3 and SmadLC. The reason of using Ub(KO) in Figure 4C (revised) is to show that the 72 kD band represents mono-ubiquitination at two different sites because if di-ubiquitination were to occur, then we would see a 63.5 kD band representing attachment of a single Ub moiety to Smad3. Short of a better substitute, we think this is a valid approach.

6. Smad3-Smad4 complexes and the role of ubiquitination (disruptase): some of the experiments using endogenous Smads are misleading. The Smad2/3 antibody is shown to precipitate lots of Smad2 but then the authors conclude that Smurf2 affects the complex of Smad3 with Smad4. Does this Smad2/3 antibody precipitate any Smad3? I think this antibody is not appropriate. Endogenous Smad3 should be precipitated with a Smad3-specific antibody, which the authors use in other figures. Figure 7B and the results importantly show that the lysine mutants (ACD and Q) cannot be phosphorylated by the receptor and thus cannot form oligomers with Smad4. How then can one use these mutants to prove that Smad3 ubiquitination in these lysines affects Smad3-Smad4 oligomerization? The ACD and Q mutations probably destroy the folding of the MH2 domain and these mutant Smad3 proteins cannot function at all. Thus the ubiquitination sites have not been proven yet and mutagenesis is not the right approach.

Mass spec is better. The authors propose that Smurf2 ubiquitinates Smad3 in the complex with Smad4 and thus acts as a disruptase. No evidence for this is shown. Are the 4 lysines of the MH2 domain available for ubiquitination in the oligomeric complex? The authors imply that they are not. Then, how do they become ubiquitinated by Smurf2? Does Smurf2 bind to Smad3 when it is in complex with Smad4? I get the idea that if the model has any relevance to the in vivo situation, Smad3 gets ubiquitinated and this prohibits oligomerization as the cartoon shows. Thus, why use the misleading term "disruptase" and discuss such a model?

We have re-done the experiments in Figure 7A using Smad3 specific antibody, as suggested and the results stay the same.

Because our new data in Figure 5E and Supplemental Fig. S5 suggest that Smad3 could be ubiquitinated in both nucleus and cytoplasmic, we have changed our wording in the manuscript, not calling Smurf2 as a "disruptase". Nevertheless, the role of Smurf2 in inhibition of Smad3 complex formation is fully vested.

We only used the Smad3 ACD or Q mutants for mapping the ubiquitin attachment sites, never intended to use them for demonstrating that they affect Smad complex formation. Although one can never be certain to separate the effect of specific replacement of a ubiquitin attachment site from misfolding of a peptidyl structure, the fact that we substituted with structurally similar amino acid residue and it is the ubiquitin-receiving lysine residue, not any other site in the vicinity, strongly argue that these lysines encompass the actual ubiquitin attachment sites. Even if we succeeded in identifying ub sites by mass spec approach, we would still be required to prove that by mutational analysis.

7. Smad nuclear shuttling and the role of Smurf2: Figure 8 uses again the Smad2/3 antibody that recognizes mainly Smad2. Yet the interperatation of these experiments is centering around Smad3! Anti-Smad3-specific antibodies need to be used for the immunofluorescence experiments. The same for figure 8B. The case is made for Smad3, yet the controls are measuring pSmad2 and Smad2. Why? pSmad3 and Smad3 should be measured. The results in page 22 top section claim that no effects on C-terminal serine phosphorylation of endogenous Smads were measured, but the figure shows only Smad2.

We have re-done the experiments in Figure 8 using Smad3 specific antibody.

Specific minor/technical comments:
1. Figure 4: many blots are over-saturated (including the long exposures but also some of the regular exposures) and this precludes rigorous evaluation of expression levels. Less saturated exposures would be better. Figure 4B lacks the control of Flag-Smad3 without Myc-Smurf2 co-transfection. Figure 4E second panel (WCL/Flag) should explain which of the multiple protein bands are specific and which are not. Figure 4F should show longer exposure of the right hand side Smad3 blot and it will probably reveal the ladder of poly-/multi-ubiquitinated Smad3 as the corresponding HA(Ub) blot on the left shows.

We have tried our best to change some of those longer exposure images with less saturated ones, but the ubiquitinated Smad3 can only be visualized on films with prolonged exposure. In those cases, both long and short exposures were shown. The control for Flag-Smad3 without Myc-Smurf2 co-transfection is now included in Figure 4C right panel. The old Figure 4E is now Fig. S2. We have marked the specific bands in the figures. Figure 4F right panel is already a longer exposure, but we did not observe any band higher than the 72KD band. The ladder of poly-ubiquitinated bands in HA(Ub) blot are likely from Smad3 associated and autoubiquitinated Smurf2 or Smad3 bound poly-ubiquitin.

2. Figure 5: panel A is saturated and it is difficult to see which are the specific bands and which are background shadows. Better contrast is needed. In panel D it is difficult to see the extra protein bands on top of the overexposed Smad3 band (Flag IP long exposure). An SDS-PAGE of better resolution is somehow needed.

We have re-done the experiments in Figure 5A, and re-adjusted the overall contrast of Figure 5D. Hopefully, the ubiquitinated Smad3 can be better seen in the revised figures.

3. Figure 6C: a lower exposure and an ubiquitin blot are needed to obtain convincing evidence about the role of the specific mutations on Smad3 ubiquitination.

The overall contrast in Figure 6C has been adjusted to enable a better visualization of the ubiquitinated Smad3 in this panel.

4. Figure 7A (Smad2 blot), 7B (Smad3 blot in Flag IP and Flag blot in WCL), 7C (both top and bottom blots) are saturated. Lower exposures are needed for accurate evaluation of differences in protein levels.

We have re-done the experiment in Figure 7A with Smad3 specific antibody. A new Figure 7A is included in the revised manuscript. Lower exposures for Figure 7C are included as well.

5. Results, page 9, start of the second paragraph: The authors describe that "it is generally believed based on previous biochemical and cell culture studies that Smurf2 regulates TGF-β signaling by targeting Smads or the type I receptor for proteasomal degradation". A review is cited for this. It will be more honest to the non-specialist reader of the paper if the term "believed" is removed. In science beliefs should not be of importance. Better to replace the word believed with the word "demonstrated" or "established". The 3 key primary references should be cited here instead of a review article: Zhang et al. 2001, Lin et al. 2000, Kavsak et al. 2000.

We have changed the wording and cited references in the revised text.

6. Discussion, page 20 middle: minor typo in the sentence: A possible explanation for this discrepancy may is that the previous study...

We corrected the typo.

2nd Editorial Decision 13 September 2011

Thank you for submitting your revised manuscript on Smad3 monoubiquitination by Smurf2 for our consideration. Two of the original referees have now assessed it once more, and I am pleased to inform you that they now consider the manuscript in principle suited for The EMBO Journal. As you will see from their comments below, they nevertheless retain a limited number of concerns remaining to be addressed before acceptance. Most importantly, points 2 and 3 of referee 1 as well as point 1 of referee 3 all refer to the new data in Figure 2H aimed at bolstering the physiological relevance of Smurf2 in TGFb-induced growth inhibition: additional replicates will be required to validate the significance of these results, while an alternative option pointed out by both referees would be to remove (and maybe only discuss) these data; in any case referee 1 also suggests to put the discussions on the possible biological importance of this new biochemical pathway into a different perspective. Related to that is referee 1's point 4, which also suggests to not overinterpret data on specific types of ubiquitination when in any case the main novel message, that Smurf2 is for the first time found to catalyze something other than degradative polyubiquitination, would not be affected by this. Finally, another essential point to clarify is referee 1's point 1 regarding the ARE-lux plasmids: I agree that the basis of these differences has to be clarified, or the data should be removed.

I am therefore returning the manuscript to you once more for a final round of minor revision. Given the time sensitivity, I hope you will be able to get a re-revised version back to us within the next four weeks (please let me know ASAP if that should pose a problem).

We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to ask you if you would be willing to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. Please let me know if you should have any additional questions regarding this re-revision or its time frame.

Sincerely,

Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors carried out a minimal revision of their paper. Overall, considering my previous comments and those of the other reviewers, I feel the paper may be appropriate. There are some residual points that require attention:

1) The ARE lux argument used in response to Rev. 1 point 1 cannot be accepted (i.e., one plasmid being responsive to Smad3 and its "putative" clone with the same name being insensitive). The authors apparently did not sequence the two plasmids to know the nature of this difference. So now that I know this, I have hard time to trust one set of data and forget about the other. Thus, the ARE experiment of Fig 2C should be removed.

2) As commented by Rev. 3, the biological relevance of Smurf2 is not coming out very impressively in this paper. The new data in Fig 2H on growth arrest is, admittedly, again very small (albeit significant). Perhaps, rather than strongly opposing what appears to me a simple fact (as I see the authors did in their rebuttal to Rev. 3), it is a better idea to comment on why loss of a single inhibitor such as Smurf2 shows limited or negligible effects on biological responses. Likely, this has to do with our rather crude way of studying growth factors responses (i.e. adding recombinant TGFb to tissue cultures). This set-up is fine for loss of positive signaling components but hardly suitable to study "extra" signaling/activity such that one emerging after loss of an inhibitor. First, we know little of what should happen by gain of endogenous TGFb signaling (a layer of regulation completely surpassed by the addition of recombinant TGFb in cultures); and second, the effect of pulsating (i.e. real/endogenous) vs constant (i.e., the only experimental one) stimulation may elicit dramatically different responses in natural tissues.
This paper stands for its biochemical characterization of monoubiquitination - multimonoubiquitination as a new layer of control for Smad activity in a genetically defined system. This is the first demonstration of this sort. That said, the authors should not push on discussing this as a key relevant step for TGFb biology.

3) to reinforce the claim that Smurf2 has biological relevance to limit TGFb growth arrest, the authors should replicate that small difference (of Fig.2H) with independent siRNAs against Smurf2 and rescue it with re-gain of Smurf2 (that should actually abolish the response to TGFb in such gain of function setting). This experiment should also erase any concern on the use of different cell populations of MEFs (as mentioned by Rev. 3). Indeed, one can always argue that long-term loss of Smurf2 may have indirect effects.
ALTERNATIVELY, the new Fig. 2H may be simply removed.

4) About monoubiquitination and oligoubiquitination and multi-monoubiquitination.
I think it is fair to state that merely judging from MW - or by using K"Zero"Ub - no solid conclusion can be taken in favor of just one of these types of modifications. It is, I feel, a rather specialistic issue, secondary to the idea (supported by the evidences here presented) that this is a non-degradative type of modification. This is a paradigm shift in the current scenario in which Smads are only envisioned as polyubiquitinated and degradated. This was an oversimplification, here rectified.

5) The authors have changed the IP set-up of Fig. 6B to avoid to show the real complexity of Smad3 ubiquitination in their deletion mutants. Hiding data is not helping their claim. In contrast, I strongly suggest to go back to the original 6B and instead better discuss about the variety of mono vs polyubiquitinated isoforms, and the potential for multiple regulatory tiers. For example, as correctly noted by another reviewer, different domains of Smad3 show differential preference of mono, oligo and polyubiquitination patterns.

Referee #2 (Remarks to the Author):

They have revised the manuscript according to this reviewer's criticisms. They failed to fully answer to my comment 3, ubiquitination may occur only a small fraction of proteins, so this may be studied in the future. I have some minor comments on this manuscript.
1. Fig. 2H. Though they tried to show the effect of Smurf2 on TGF-b-induced growth inhibition (according to the comment 5 by reviewer 1), differences between Smurf2- and Smurf2+ cells were very small. I do not know whether this information is necessary in this manuscript.
2. Page 14, line 2 from the bottom. Linker phosphorylation is not shown in "Fig. 4C".
3. Page 18, line 7. Fig. 7B may show the interaction of Flag-Smad3 with endogenous Smad4. But it is labeled in the figure that "Myc-Smad4" is transfected. Which is true?

2nd Revision - authors' response 05 October 2011

Responses to reviewers:

We thank the reviewers again for their effort in evaluating our manuscript and for their constructive comments. Here are our responses to comments raised by reviewers.

Reviewer 1:

1) The ARE lux argument used in response to Rev. 1 point 1 cannot be accepted (i.e., one plasmid being responsive to Smad3 and its "putative" clone with the same name being insensitive). The authors apparently did not sequence the two plasmids to know the nature of this difference. So now that I know this, I have hard time to trust one set of data and forget about the other. Thus, the ARE experiment of Fig 2C should be removed.

Both ARE-Lux plasmids were deposited in Addgene (cat # 11768 and cat# 14934) and sequenced by Addgene. Based on the sequencing results, these two plasmids have a 200 bp difference within their multiple cloning regions flanking the 3XARE. Without additional detailed deletion and mutagenesis analysis, we could not identify the nature of the one responsible for Smad dependence. Since we decide to follow the reviewersí recommendation of focusing on the main point of this study, which is the non-degradative ubiquitination of Smads by Smurf2, we have removed the old Fig. 2C regarding the ARE-Lux reporter assay. Now, the main focus of this section is devoted to Smurf2 regulation of Smad-dependent transcription per se, rather than pin-pointing down the specificity between Smad2 and Smad3.

2) As commented by Rev. 3, the biological relevance of Smurf2 is not coming out very impressively in this paper. The new data in Fig 2H on growth arrest is, admittedly, again very small (albeit significant). Perhaps, rather than strongly opposing what appears to me a simple fact (as I see the authors did in their rebuttal to Rev. 3), it is a better idea to comment on why loss of a single inhibitor such as Smurf2 shows limited or negligible effects on biological responses. Likely, this has to do with our rather crude way of studying growth factors responses (i.e. adding recombinant TGFb to tissue cultures). This set-up is fine for loss of positive signaling components but hardly suitable to study "extra" signaling/activity such that one emerging after loss of an inhibitor. First, we know little of what should happen by gain of endogenous TGFb signaling (a layer of regulation completely surpassed by the addition of recombinant TGFb in cultures); and second, the effect of pulsating(i.e. real/endogenous) vs constant (i.e., the only experimental one) stimulation may elicit dramatically different responses in natural tissues.
This paper stands for its biochemical characterization of monoubiquitination - multimonoubiquitination as a new layer of control for Smad activity in a genetically defined system. This is the first demonstration of this sort. That said, the authors should not push on discussing this as a key relevant step for TGFb biology.

We agree with the reviewers that our current methods of interrogation might not suitable to study the inhibitory role of Smurf2 in TGF- biology; and decided not to over-interpret the small difference of TGF- -induced growth inhibition between the presence and absence of Smurf2. Instead, we follow the reviewersí recommendation of focusing on the biochemical characterization

of ubiquitination of Smad3 by Smurf2. We have modified the relevant part of the text.

3) to reinforce the claim that Smurf2 has biological relevance to limit TGFb growth arrest, the authors should replicate that small difference (of Fig.2H) with independent siRNAs against Smurf2 and rescue it with re-gain of Smurf2 (that should actually abolish the response to TGFb in such gain of function setting). This experiment should also erase any concern on the use of different cell populations of MEFs (as mentioned by Rev. 3). Indeed, one can always argue that long-term loss of Smurf2 may have indirect effects.
ALTERNATIVELY, the new Fig. 2H may be simply removed.

The effect of Smurf2 on TGF-b-mediated growth arrest is indeed small but repeatable in primary MEFs. The reviewerís suggestion with independent siRNA against Smurf2 and rescue with regain of Smurf2 is valid but unfeasible in primary MEFs. Since we decide to follow the reviewersí recommendation of focusing on the main point of this study, which is the non-degradative ubiquitination of Smads by Smurf2, we have removed the old Fig. 2H for future characterization of biological significance of the described Smurf2 function.

4) About monoubiquitination and oligoubiquitination and multi-monoubiquitination.
I think it is fair to state that merely judging from MW - or by using K"Zero"Ub - no solid conclusion can be taken in favor of just one of these types of modifications. It is, I feel, a rather specialistic issue, secondary to the idea (supported by the evidences here presented) that this is a non-degradative type of modification. This is a paradigm shift in the current scenario in which Smads are only envisioned as polyubiquitinated and degradated. This was an oversimplification, here rectified.

We agree with the reviewersí assessment that the types of ubiquitin modification afford to Smad3 by Smurf2 are of specialistic issue. So, we modified the text in a way that while stating the predominant form of modification is mono-ubiquitination, we acknowledge that other forms of oligo-ubiquitination also exist, but the length of ubiquitin chains is not sufficiently long enough to mark for proteasome degradation.

5) The authors have changed the IP set-up of Fig. 6B to avoid to show the real complexity of Smad3 ubiquitination in their deletion mutants. Hiding data is not helping their claim. In contrast, I strongly suggest to go back to the original 6B and instead better discuss about the variety of mono vs polyubiquitinated isoforms, and the potential for multiple regulatory tiers. For example, as correctly noted by another reviewer, different domains of Smad3 show differential preference of mono, oligo and polyubiquitination patterns.

We have added back the FLAG IP/HA(Ub) blot in the Fig 6B, and modified the text accordingly.

Reviewer 2:

1. Fig. 2H. Though they tried to show the effect of Smurf2 on TGF-b-induced growth inhibition (according to the comment 5 by reviewer 1), differences between Smurf2- and Smurf2+ cells were very small. I do not know whether this information is necessary in this manuscript.

See our response to reviewer 1, we have removed Fig. 2H.

2. Page 14, line 2 from the bottom. Linker phosphorylation is not shown in "Fig. 4C".

The linker phosphorylation is shown in Fig 4D. We have corrected this mistake.

3. Page 18, line 7. Fig. 7B may show the interaction of Flag-Smad3 with endogenous Smad4. But it is labeled in the figure that "Myc-Smad4" is transfected. Which is true?

We have corrected this mistake in the text.

