Fine-tuning BMP7 signalling in adipogenesis by UBE2O/E2-230K-mediated monoubiquitination of SMAD6

Xiaofei Zhang, Juan Zhang, Andreas Bauer, Long Zhang, Douglas W. Selinger, Chris X. Lu and Peter ten Dijke

Corresponding author: Peter ten Dijke, Leiden University Medical Center

Review timeline:

Submission date: 13 September 2012
Editorial Decision: 24 October 2012
Revision received: 31 January 2013
Accepted 01 February 2013

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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 24 October 2012

Thank you very much for submitting your paper on the role of UBE2O in regulating Smad6 for consideration to The EMBO Journal editorial office. I apologize for the delay in my response caused by rather late incoming assessments. Indeed, a third scientist that originally agreed to referee your study did never return any comments.

You will recognize that both scientists express principle interest in your data. They ask for some further experimental demands to specify the type of ubiquitin-linkage (mono- versus poly-ubiquitination). Rather more importantly, both referees request better physiological relevance/evidence for BMP-dependent UBE2O regulation.

Conditioned on this, we would like to offer you the opportunity to revise the current dataset. Please do not hesitate to contact me with in case of further questions or to outline the timeframe for necessary experimental work (preferably via E-mail).

I hope that precise communication of the expectations from our expert referees facilitates efficient proceedings for your study. I do also have to remind you that the final decision on further proceedings at The EMBO Journal depends on the completeness of the revised paper.

REFEREE REPORTS:
Referee #1:

The new paper by Zhang et al from Peter ten Dijke's lab makes the first molecular link between the ubiquitin protein ligase UBE2O and the regulatory protein Smad6 that blocks signal transduction by bone morphogenetic (BMP) receptors. UBE2O is also shown to act as hybrid E2-E3 ligase that mono-ubiquitylates Smad6, thus limiting the association of Smad6 with BMP type I receptors. Based on this molecular mechanism, UBE2O and Smad6 mono-ubiquitylation are shown to enhance BMP signaling and BMP-induced cell differentiation. The role of UBE2O as a novel regulator of BMP signaling is examined to some level but no circumstances that regulate the activity or association of UBE2O to Smad6 are described. This leaves an important caveat about the physiological setting during which Smad6 ubiquitylation by UBE2O takes place.

The Smad6-TAP experiment identified numerous proteins and abundantly found almost every protein of the ribosome. Did the whole ribosome co-purify with Smad6? This is worth discussing briefly. If this is so, then Figure 1A can be annotated with an extra line pointing to the ribosome. In Figure 1C, the pattern of Flag-Smad6 in the TCL is almost identical to the pattern of UBE2O in the IP (double band). Is this a coincidence? The pattern of F-Smad6 in the IP and the UBE2O in the TCL do not show the same double band. The reading of the paper would be facilitated if Figure S1 is transposed to the main figure 1. Space limitation is not a problem.

Is the Smad6 mono-ubiquitylation modulated by BMP signaling? Can the Smad6-Ubi protein pool be identified in cells responding to BMP without transfecting Smad6 or ubiquitin? If Smad6 gets mono-ubiquitylated and Smad7 gets poly-ubiquitylated (Figure S2) what defines the specificity of these two patterns of ubiquitylation? Does UBE2O enhance the activity of another ubiquitin ligase that regulates Smad7 such as Arkadia/RNF12 as the authors discuss? Figures S2 and S3 can be transposed to the corresponding main figures to facilitate reading. The chimeric Smad6 proteins (Figure S3C) are not explained. What are the 1, 2, 3, 4 fragments? What does L stand for? I guess W stands for wild type. Figure S3D raises the question of what is the definition of mono-ubiquitylation and poly-ubiquitylation. Are the high molecular size ubiquitin smears observed in all gels that have not been trimmed irrelevant? Do some of the chimeric proteins become more heavily poly-ubiquitylated? Does this ubiquitylation have any impact on Smad6 protein stability?

The ubiquitylated Smad6 is shown to be mainly cytoplasmic. This is because UBE2O mainly localizes in the cytoplasm (Figure 6A). This is relevant to the major comment raised in the beginning and so the fractionation experiment is important to be shown with endogenous proteins. This is because it relates to the model of how UBE2O acts on Smad6 and what is the relationship of this action during BMP signaling.

The specificity of the Smad6 K174R mutant is quite remarkable (Figure S4). Since this is explained by the association of Smad6 with BMP type I receptors (Figure S6), and Smad6 binds to ALK2 and ALK6, is it possible that the cells examined in the signaling experiments (293T, HeptG2) express only ALK2 and not ALK6? If this is so, can Smad6K174R be more inhibitory against BMP2 and BMP6 that signal via ALK6 in a cell model that expresses ALK6?

The Smad6 knockout MEF experiment is quite intriguing (Figure 7G) but generates ambiguity as the effects are very small. Can it be for example that effects of UBE2O in WT MEFs are stronger because levels of UBE2O are higher? Statistical analysis of these data seems to be important after normalization for expression levels of Smad1 and the transfected UBE2O.

Minor formatting comments:
The paper is in need of revision for scientific and syntactical accuracy. A single example from page 13, end of Discussion where "the kinase-inactive UBE2O" probably refers to "catalytically-inactive UBE2O". Such minor but important typographical errors are abundant.

The list of plasmids does not seem to include the Flag-Smad6-ubiquitin fusion. Construction of this plasmid is only minimally described. In association to the supplementary plasmid list, a more detailed plasmid construction method can be presented in the supplemental part for the various new constructs.
The RT-PCR graphs would appear better if the y-axis (level of PCR signal) was labelled. Adi(day) stands for adipocytes (day)?

Referee #2:

Zhang et al reported that UBE2O is a novel E2-E3 hybrid ubiquitin enzyme for Smad6 monoubiquitination. They identified the specific cysteine site for its E2 function and the specific lysine residue on Smad6 monoubiquitination. They further demonstrated how the monoubiquitination affects the functions of Smad6 in BMP signaling, specifically how the enzyme and monoubiquitinated Smad6 regulates BMP7-induced adipogenesis in C3H10T1/2 cells.

Overall, this is a very novel and interesting study. There are two novel points: 1. Smad6 is mono-ubiquitinated, which inhibits its function as a BMP inhibitor; 2. Ube2O is an E2-E3 hybrid enzyme for ubiquitin conjugation. The study offers a new look at the role of Smad6 in BMP signaling. The novelty and scientific advancement of the study is suitable for publication in EMBO J.

However, I have a few concerns.

Specific points:

1. In some western blots, image quality of anti-Smad1 is poor, for example Fig 5B, 7B/D/G. These should be improved.
2. Fig. 6: a better way to distinguish the binding of un-modified or modified Smad6 to receptor is to have a good/high modification (e.g. Fig. 2A, Flag/Flag panel), and then use the receptor to pull down the mixture. Will the upper band lose binding? Fig 6 also needs some comparisons. For example, KR mutant, and WT in the presence/absence of Ube2O and/or Ubi. This reviewer is a bit confused with Fig. 6B/C. Is Smad6-Ubi the fusion? If so, the text should clearly state that Smad6-Ubi is a fusion, not mono-ubiquitinated Smad6. If not, why are there two bands? If the upper band has a weak binding, why is the lower band weak too?
3. Specificity: how does the monoubi-Smad6 only affect the BMP7 signaling, not BMP2/6 signaling? Does Smad6 bind to Alk2 vs Alk6 differently? Fig. S6B: no Smad6-Alk3 interaction, is it true?
4. Fig 2B and 2C: why did Ni-NTA pull down ubi-Smad6 as well as Smad6? Or is it just a non-specific band?
5. Fig 3B: it is not quite clear about Deletion 1 and Deletion 2. Put the info in here so readers do not have to visit the supplemental data.
6. Page 13, line 3, "kinase-dead" should be E2 or E3-dead (whichever is correct).

Additional comments (this should be discussed):
1. It will be very helpful to see Smad6 monoubiquitination via mass spec analysis.
2. Are there any pathological conditions under which abnormal Ube2O level is associated with aberrant BMP signaling?
3. BMPs broadly regulate cellular functions. It would be nice to include another assay (besides adipogenesis), for example, BMP2-induced osteoblast differentiation in C2C12.
4. Is there any other substrate for UBE-2O? What's its specificity towards Smad6?
5. Where is Ube2O localized? Where do the Ube2O and Smad6 interact?

1st Revision - authors' response 31 January 2013

We are grateful for the insightful and helpful comments on our manuscript entitled "Fine-tuning BMP7 signalling in adipogenesis by UBE2O/E2-230K-mediated monoubiquitination of SMAD6" by Zhang et al. Please see the rebuttal text document for an itemized report on how we have addressed all of the comments. We think the revision has strongly improved our manuscript and hope you find it now acceptable for publication in EMBO J.
Reviewer 1

The new paper by Zhang et al from Peter ten Dijke's lab makes the first molecular link between the ubiquitin protein ligase UBE2O and the regulatory protein Smad6 that blocks signal transduction by bone morphogenetic (BMP) receptors. UBE2O is also shown to act as hybrid E2-E3 ligase that mono-ubiquitylates Smad6, thus limiting the association of Smad6 with BMP type I receptors. Based on this molecular mechanism, UBE2O and Smad6 mono-ubiquitylation are shown to enhance BMP signalling and BMP-induced cell differentiation. The role of UBE2O as a novel regulator of BMP signalling is examined to some level but no circumstances that regulate the activity or association of UBE2O to Smad6 are described. This leaves an important caveat about the physiological setting during which Smad6 ubiquitylation by UBE2O takes place.

Response: Our findings that knockdown of UBE2O inhibits SMAD6 monoubiquitination (Figure 2D) and BMP7-induced responses (Figure 7A and 7B) are illustrative for the physiological relevance of UBE2O in mediating SMAD6 monoubiquitination and BMP signalling. Furthermore, in response to reviewer comments we have examined the kinetics of expression levels of UBE2O, SMAD6 and phospho-SMAD1 during the commitment of mesenchymal stem cells to pre-adipocytes, which is strongly stimulated by BMP7, and the subsequent differentiation into adipocytes that occurs in the absence of exogenous BMP7 by placing the cells under adipogenic culture conditions. We found that UBE2O and SMAD6 are both upregulated with different kinetics during the commitment to pre-adipocytes, in that peak of UBE2O expression was preceding the peak of SMAD6 expression. Thus, UBE2O may function as a positive feedback regulator for early stage of BMP7-induced commitment to pre-adipocytes by inhibiting the inhibitory effects of SMAD6. In the differentiation towards to mature adipocytes the UBE2O expression is low as no enhancement of BMP signalling during that phase is needed. Previous reports have shown that endogenous BMP signalling is higher in proliferating pre-adipocytes, but lower in differentiating adipocytes (Suenaga et al, 2010). Thus, BMP signalling needs to be temporally enhanced during adipocyte differentiation. Our results suggest UBE2O might help to control BMP7-induced adipocyte differentiation through SMAD6. We now have added this data into our manuscript as Figure 5E and 5F, and we now have discussed it in the text.

The Smad6-TAP experiment identified numerous proteins and abundantly found almost every protein of the ribosome. Did the whole ribosome co-purify with Smad6? This is worth discussing briefly. If this is so, then Figure 1A can be annotated with an extra line pointing to the ribosome.

Response: The reason why we didn’t add ribosome proteins in our graph is that we also found these ribosome proteins in the interactome with other “bait proteins”. We now have mentioned this issue in materials and methods.

In Figure 1C, the pattern of Flag-Smad6 in the TCL is almost identical to the pattern of UBE2O in the IP (double band). Is this a coincidence? The pattern of F-Smad6 in the IP and the UBE2O in the TCL do not show the same double band.

Response: The pattern of the F-SMAD6 in the IP and the UBE2O in the TCL is indeed a coincidence. Please see the original blot of these experiments (source data Figure 1). We transfected very little amount of SMAD6 for the IP experiments and the exposure time for this blot is also short. These two bands merge to one band if the expression of SMAD6 is stronger or the exposure time prolonged (SMAD6 after IP experiments is too strong to be seen two bands). The upper band could correspond to phosphorylated SMAD6; SMAD6 was shown to be phospho-protein (Glesne & Huberman, 2006). Whether UBE2O is under any post-translation modification is unknown. However, we found this modification is more obvious in UBE2O deletion 1 (Figure S1B) and in co-transfection of UBE2O with ARKADIA (Figure S6A).
The reading of the paper would be facilitated if Figure S1 is transposed to the main figure 1. Space limitation is not a problem.

Response: As requested by the reviewer we have transposed Figure S1 to main Figure 1.

Is the Smad6 mono-ubiquitylation modulated by BMP signalling? Can the Smad6-Ubi protein pool be identified in cells responding to BMP without transfecting Smad6 or ubiquitin?

Response: As requested by the reviewer we have performed the ubiquitination assay for SMAD6 in the absence or presence of BMP or TGF-b (Figure R1). We were unable observe significant differences of monoubiquitinated SMAD6 upon BMP or TGF-b challenge. Also, we didn’t observe monoubiquitinated SMAD6 in cells treated with BMP7 without transfecting SMAD6 or ubiquitin (unpublished observations). We speculate that other stimuli, not BMP and TGF-b, may response for activating UBE2O.

Figure R1 HEK293T cells were transfected with Flag-SMAD6 (2.5 ug), very little amount of His-ubiquitin (0.2 ug) and UBE2O-Myc (2.5 ug) for 48 h. Cells were treated as indicated (5 ng/ml TGF-β3, 20ng/ml BMP4 and 100 ng/ml BMP7) for 1 h. In vivo ubiquitination of SMAD6 was performed by immunoprecipitation. Cells were sonicated with 1% SDS, then diluted to 0.1% SDS for immunoprecipitation with anti-Flag resin. Monoubiquitinated SMAD6 was detected by ubiquitin antibody. IP, immunoprecipitation; IB, immunoblotting; TCL, total cell lysate.

If Smad6 gets mono-ubiquitylated and Smad7 gets poly-ubiquitylated (Figure S2) what defines the specificity of these two patterns of ubiquitylation? Does UEB2O enhance the activity of another ubiquitin ligase that regulates Smad7 such as Arkadia/RNF12 as the authors discuss?

Response: We speculated that the E3 enzyme defines the specificity of mono-/polyubiquitination. To confirm this hypothesis, we first performed the interaction between UBE2O and
ARKADIA/RNF12 (Figure S6A). UBE2O was found to strongly interact with ARKADIA or RNF12. Ubiquitination assay for SMAD7 demonstrated that UBE2O potentiates ARKADIA- or RNF12-induced polyubiquitination (Figure S6B and S6C). In conclusion, by recruiting E3 ligases ARKADIA or RNF12, UBE2O can stimulate SMAD7 polyubiquitination. This issue has now been added as Figure S6 and discussed in the text.

Figures S2 and S3 can be transposed to the corresponding main figures to facilitate reading.

Response: As requested by the reviewer we have transposed Figure S2 and S3 to Figure 2 and Figure 4 accordingly.

The chimeric Smad6 proteins (Figure S3C) are not explained. What are the 1, 2, 3, 4 fragments? What does L stand for? I guess W stands for wild type.

Response: We have clarified this in the legend of Figure 4A.

Figure S3D raises the question of what is the definition of mono-ubiquitylation and poly-ubiquitylation. Are the high molecular size ubiquitin smears observed in all gels that have not been trimmed irrelevant? Does some of the chimeric proteins become more heavily poly-ubiquitylated? Does this ubiquitylation have any impact on Smad6 protein stability?

Response: We do see more smears (polyubiquitinated SMAD6) for some chimeric SMAD6 proteins. This might come from the fact that ubiquitin could shift to neighbouring lysine residues or other amino acids when some lysines are mutated (Hagai et al, 2012; Tokarev et al, 2011; Wang et al, 2007). However, we found that UBE2O only very weakly contributed to the polyubiquitination of wild-type SMAD6 (Figure 2A). Also, we did not observe noticeable changes of SMAD6 protein stability (see analysis of total cell lysis of Figure 4C).

The ubiquitylated Smad6 is shown to be mainly cytoplasmic. This is because UBE2O mainly localizes in the cytoplasm (Figure 6A). This is relevant to the major comment raised in the beginning and so the fractionation experiment is important to be shown with endogenous proteins. This is because it relates to the model of how UBE2O acts on Smad6 and what is the relationship of this action during BMP signalling.

Response: As requested by reviewer we performed the western blot for four proteins which are markers for cytoplasmic extracts, membrane extracts, nuclear soluble extracts and chromatin binding extracts. These results were included in Figure 6A.

The specificity of the Smad6 K174R mutant is quite remarkable (Figure S4). Since this is explained by the association of Smad6 with BMP type I receptors (Figure S6), and Smad6 binds to ALK2 and ALK6, is it possible that the cells examined in the signalling experiments (293T, HepG2) express only ALK2 and not ALK6? If this is so, can Smad6K174R be more inhibitory against BMP2 and BMP6 that signal via ALK6 in a cell model that expresses ALK6?

Response: As requested by reviewer, we depleted ALK6 in HEPG2 cells by two independent shRNAs. The efficiency of knockdown was checked by real-time PCR. ALK2 expression was checked as control for the specificity of shRNAs targeting ALK6. BMP2 (B), BMP6 (C) and BMP7 (D) induced BRE-luciferase activity was analysed in ALK6 depleted and control shRNA infected
cells (Figure R2). No significant difference was observed in the inhibitory ability of SMAD6 wild-type or K174R on BMP2/6-induced BRE-Luc activity in ALK6 knockdown cells.

Figure R2

![Figure R2](image)

Figure R2: (A) Real-time PCR was used to detect ALK6 or ALK2 mRNA level in HEPG2 cells transduced with ALK6 shRNAs (TRCN000000451 (sh1) and TRCN000000454 (sh2)) viruses. (B), (C) and (D) HEPG2 cells expressing ALK6 shRNA were transfected with BRE-Luc reporter and SMAD6 or K174R for 24 h. Luciferase activity was analysed after cells were stimulated with 50 ng/ml BMP2 (B), 50 ng/ml BMP6 (C) or 50ng/ml BMP7 (D) overnight. NS represents non-specific shRNA.

The Smad6 knockout MEF experiment is quite intriguing (Figure 7G) but generates ambiguity as the effects are very small. Can it be for example that effects of UBE2O in WT MEFs are stronger because levels of UBE2O are higher? Statistical analysis of these data seems to be important after normalization for expression levels of Smad1 and the transfected UBE2O.

Response: We analysed the expression of UBE2O in wild-type and SMAD6 knockout MEF cells by ImageJ (NIH). There is no obvious difference of UBE2O expression in these two cells (Figure R3). The expression of phosphorylated SMAD1 have been re-analysed and Student’s t test was used for statistical analysis and p<0.05 was considered to be statistically significant. Figure 7J was changed accordingly.
Figure R3 The relative UBE2O expression level in SMAD6 wild-type or knockout MEF cells (Figure 7G) (normalised to Actin) was quantified. No significant differences were observed.

Minor formatting comments: The paper is in need of revision for scientific and syntactical accuracy. A single example from page 13, end of Discussion where "the kinase-inactive UBE2O" probably refers to "catalytically-inactive UBE2O". Such minor but important typographical errors are abundant. The list of plasmids does not seem to include the Flag-Smad6-ubiquitin fusion. Construction of this plasmid is only minimally described. In association to the supplementary plasmid list, a more detailed plasmid construction method can be presented in the supplemental part for the various new constructs. The RT-PCR graphs would appear better if the y-axis (level of PCR signal) was labelled. Adi(day) stands for adipocytes (day)?

Response: We apologize for these mistakes and have corrected them and changed the format according to reviewer suggestion. Details regarding the plasmid construction method have been added in “Plasmids” of Supplementary Materials and Methods. Graph of all RT-PCR results has been labelled with relative mRNA expression level and adi(day) has been explained in Figure legends of Figure 5D an Figure 7F.

Referee #2: Zhang et al reported that UBE2O is a novel E2-E3 hybrid ubiquitin enzyme for Smad6 monoubiquitination. They identified the specific cysteine site for its E2 function and the specific lysine residue on Smad6 monoubiquitination. They further demonstrated how the monoubiquitination affects the functions of Smad6 in BMP signalling, specifically how the enzyme and monoubiquitinated Smad6 regulates BMP7-induced adipogenesis in C3H10T1/2 cells. Overall, this is a very novel and interesting study. There are two novel points: 1. Smad6 is monoubiquitinated, which inhibits its function as a BMP inhibitor; 2. Ube2O is an E2-E3 hybrid enzyme for ubiquitin conjugation. The study offers a new look at the role of Smad6 in BMP signalling. The novelty and scientific advancement of the study is suitable for publication in EMBO J. However, I have a few concerns.

Response: Many thanks for these positive remarks, please see our point by point answer to your comments and criticisms.

Specific points:
1. In some western blots, image quality of anti-Smad1 is poor, for example Fig 5B, 7B/D/G. These should be improved.
Response: We agree with the reviewer and we have repeated the western blot for SMAD1 with new antibody. Blots for SMAD1 have been changed accordingly.

2. Fig. 6: a better way to distinguish the binding of un-modified or modified Smad6 to receptor is to have a good/high modification (e.g. Fig. 2A, Flag/Flag panel), and then use the receptor to pull down the mixture. Will the upper band lose binding? Fig.6 also needs some comparisons. For example, KR mutant, and WT in the presence/absence of Ube2O and/or Ubi. This reviewer is a bit confused with Fig. 6B/C. Is Smad6-Ubi the fusion? If so, the text should clearly state that Smad6-Ubi is a fusion, not mono-ubiquitinated Smad6. If not, why are there two bands? If the upper band has a weak binding, why is the lower band weak too?

Response: When we used the purified receptor (HA-caALK2, Figure 6B) to perform the immunoprecipitation assay, the upper band (monoubiquitinated SMAD6) is still present but the interaction between receptor and monoubiquitinated SMAD6 is much less compared with the interaction between receptor and non-modified SMAD6 (Figure 6B). This can be explained by the fact that SMAD6 forms homomeric complexes (Table S1).

Monoubiquitinated SMAD6 induced by UBE2O and Ubiquitin has a predominantly localisation in the cytoplasm as shown in Figure 6A (lane anti-HA after IP). However, most SMAD6 without modification is presented in all subcellular as showed by Figure 6A (lane anti-Flag after IP) and Figure S4B. The KR mutant showed the similar localization pattern as the wild-type SMAD6 (unpublished observations). We think it’s reasonable since the relative amount of SMAD6 that is monoubiquitinated is little.

The Figure 6B and 6C are in vitro binding assays. All proteins that are shown in these 2 panels were purified from HEK293T cells. The SMAD6-ubi represents real monoubiquitinated-SMAD6 (not artificial SMAD6-ubiquitin fusion protein in Figure S4B. We have now clarified this in the Figure legend of Figure 6B and we changed its description to Flag-Ubi-SMAD6. The latter protein was purified from the HEK293T cells transfected with SMAD6, UBE2O and ubiquitin.

The reason for two bands upon purification of monoubiquitinated SMAD6 comes from the ability of SMAD6 to self interact; non-monoubiquitinated SMAD6 co-purifies with monoubiquitinated SMAD6. The association of monoubiquitinated SMAD6 and unmodified SMAD6 may trigger the cytoplasmic localisation of unmodified SMAD6. This may explain why both bands are weaker.

3. Specificity: how does the monoubi- Smad6 only affect the BMP7 signalling, not BMP2/6 signalling? Does Smad6 bind to Alk2 vs Alk6 differently? Fig. S6B: no Smad6-Alk3 interaction, is it true?

Response: We have screened all signalling pathways affected by SMAD6 and have only found differences in BMP7-induced signalling activation. We speculate that the conformation changes of monoubiquitinated SMAD6 make it have less binding affinity to ALK2, but not to ALK6. When we performed the immunoprecipitation assays in transfected cells, we didn’t observe any differences between SMAD6 binding with ALK2 and binding with ALK6. When we used a long time exposure, we did see the interaction between SMAD6 and ALK3. However, the interaction is weaker compared with the interaction between SMAD6 and ALK2/6.

4. Fig 2B and 2C: why did Ni-NTA pull down ubi-Smad6 as well as Smad6? Or is it just a non-specific band?

Response: When we performed the Nickel pull down assay, there is always a specific band. We speculate that the Nickel beads bind to and pull down the non-modified SMAD6 through the histidine within SMAD6 even we washed the nickel beads with 20 mM imidazole.
5. Fig 3B: it is not quite clear about Deletion 1 and Deletion 2. Put the info in here so readers do not have to visit the supplemental data. 6. Page 13, line 3, "kinase-dead" should be E2 or E3-dead (whichever is correct).

Response: As requested by reviewer we have added the missing information about deletions of UBE2O in Figure 1F. “kinase-dead” is changed to catalytically inactive. We apologize for this mistake.

Additional comments (this should be discussed): 1. It will be very helpful to see Smad6 monoubiquitination via mass spec analysis. 2. Are there any pathological conditions under which abnormal Ube2O level is associated with aberrant BMP signalling? 3. BMPs broadly regulate cellular functions. It would be nice to include another assay (besides adipogenesis), for example, BMP2-induced osteoblast differentiation in C2C12. 4. Is there any other substrate for UBE-2O? What's its specificity towards Smad6? 5. Where is Ube2O localized? Where do the Ube2O and Smad6 interact?

Response: All these issues now have been discussed as requested by reviewer.

1. We agree with the reviewer. It would be very helpful to see monoubiquitinated SMAD6 via mass spectral analysis. However, we find this beyond the scope of the present paper, and would like to report on that in a future study. We now have discussed it in the text.

2. We now have shown that both UBE2O and SMAD6 are upregulated in BMP7 induces commitment of mesenchymal stem cells to pre-adipocytes (see also response to reviewer 1, page1). UBE2O may function as a positive feedback regulator for early stage of BMP7-induced adipocyte differentiation by inhibiting the inhibitory effects of BMP7-induced SMAD6. These results is consistent with previous report that endogenous BMP signalling is higher in proliferating pre-adipocytes, but lower in differentiating adipocytes (Suenaga et al, 2010), which suggest that BMP signalling needs to be tightly controlled during adipocyte differentiation. Our results suggest UBE2O might help to control BMP7 induced adipocyte differentiation through SMAD6. We now have added it as Figure 5E and 5F and have discussed it in the text.

3. BMPs play an important role in promoting osteoblast differentiation. Forced expression of UBE2O was also found to promote BMP6-induced SMAD1 phosphorylation and alkaline phosphatase activity (an early marker for osteoblast differentiation) in C2C12 cells (Figure 7G and 7H). We now have discussed it in the text.

4. We speculated that the E3 enzyme defines the specificity of mono-/polyubiquitination. We have shown that the polyubiquitination of SMAD7 is also regulated by UBE2O (Figure 2A). By recruiting RNF12 or ARKADIA, UBE2O promotes SMAD7 polyubiquitination (see our response to reviewer 1 and Figure S6). It has been reported that UBE2O interacts with more than 100 E3s (Markson et al, 2009). In our current research, UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6, which does not require the recruitment of separate E3 ubiquitin ligase.

5. We performed cell fractionation assay and found that UBE2O is mainly localised in the cytoplasm. Moreover, the interaction of UBE2O and SMAD6 mainly occurs in the cytoplasm (Figure 6A).
References

Glesne D, Huberman E (2006) Smad6 is a protein kinase X phosphorylation substrate and is required for HL-60 cell differentiation. Oncogene 25: 4086-4098


Accepted 01 February 2013

The paper has been re-reviewed by one original referee with no further comments.