SCFFBXL15 regulates BMP signaling by directing the degradation of HECT-type ubiquitin ligase Smurf1

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1st Editorial Decision 09 March 2010

Thank you for submitting your research manuscript to The EMBO Journal editorial office. The work has been seen by three expert referees who's assessment you will find enclosed below for your information. As you will see, refs#1 and #2 appreciate some interest in your findings whereas ref#3 does find the conceptual insight rather limited. Careful reading of all the reports reveals that apart from this conceptual concern, the experimental evidence and in particular physiological significance of the proposed regulation of Smurf1 by FBXL15 remains currently uncertain. As this is a rather important measure at least from the perspective and scope of our more general and very competitive journal, I am very sorry to have to communicate that we all had to conclude that we are unable to offer any further proceedings at this preliminary state of analyses.

Please understand that The EMBO Journal demands complete papers describing original research of general rather than specialist interest in molecular biology that need urgent publication because they report novel findings of wide biological significance and a sufficient level of molecular understanding. We are therefore only able to pursue manuscripts that receive enthusiastic support from at least the majority of our referees during initial review. With this being not the case for your current submission I am sorry to have to return the paper to you at this point with the message that we are unable to offer further proceedings.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.
Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this paper, Cui et al; present a series of experiments that address the potential role of FBXL15 in the degradation of Smurf1 and Smurf2 through the SCFFBXL15 mediated complex. This is a novel study demonstrating that turnover of a HECT type E3 enzyme could be regulated in trans by a SCF complex. While the findings are of potential interest in HECT E3 regulation, there are several concerns I have regarding the experiments and the data interpretation.

Major issues:

Fig 1 B: The authors demonstrate that FBXL15 belongs to the SCF complex composed by Cullin1, Skp1 and Roc1. To prove this hypothesis, the authors over-expressed these different proteins and show that they can be pulled down by GST-FBXL15 wt, but not with the delta-F mutant. In order to be confident with the kind of SCF complex we are dealing with, this observation needs to be confirmed by co-immunoprecipitation, ideally with endogenous proteins.

Fig 1 C: The authors show that over-expression of FBXL15 down-regulates the expression of Smurf1 wt and also the Smurf1 CA mutant. They conclude that FBXL15 induced degradation is beyond Smurf1 activity. However, on the western-blot, we clearly see that Smurf1 CA mutant is less downregulated than the wild-type by FBXL15. The authors should quantify the blots and provide some statistics. If the inactive mutant is less suppressed, it will be necessary to provide some explanations. For example they should test if the CA mutant is less well interacting with FBXL15 or is less ubiquitylated. Or they may want to carry out a dose response of FBXL15.

Fig. 1G is not very convincing. Please provide quantification and statistics.

Fig 2A: The authors have to show the immunoblot of FBXL15 to evaluate the efficiency of FBXL15 silencing in this experiment.

Fig 2A and 2B: The half life of Smurf1 alone is approximately 11 hours (Fig2B). When Smurf1 is co-transfected with a control siRNA which is supposed to not disturb the protein expression; the half life of the protein falls to approximately 6 hours (Fig2A). This discrepancy is surprising, because the addition of FBXL15 siRNA restores the half-life of Smurf1 to 12 hours which is approximately the same as Smurf1 alone (Fig2B).

Fig 4A: Using different fragments of Smurf1, the authors find that the region 234-372 contains the signal for FBXL15 degradation. However, the study is not complete; they have to provide us also with the fragment aa 1 to 234 corresponding to the C2 domain.

Fig 4 C and D: The authors mention in the text that Smurf1 mutant K357R is "completely resistant to the degradation by FBXL15". It is clear that this mutation decreased the effect of FBXL15 on Smurf1, however this decreases seems to be not totally blocked. To give further evidence for this hypothesis, the authors have to assess the half-life of Smurf1, Smurf1 K357R, and finally with Smurf K357R + FBXL15. The blots have to be quantified.

Fig. 4E. In vitro ubiquitinations are not very specific. The authors should test the ubiquitination of Smurf1 in Hek293 cells, as they have done in Fig. 2C.

Fig 5: In this figure the authors show that Smurf1 and 2, but not the other Nedd4 related enzymes
are a target of FBXL15. They do that by co-ips, in which they find that all the Nedd4 related enzymes interact with FBXL15. Interestingly there are differences, as Smurf1 and 2, but even more so NEDL1, NEDL2, bind stronger, and Nedd4-1 and Nedd4-2 show weaker binding. Then they test the effect of FBXL15 on stability of 4 of these enzymes (Smurf1 and 2; Nedd4-1 and AIP4), and find that only Smurf1 and 2 are affected. With the same enzymes they carry out in vitro ubiquitination assays. However, from these experiments one cannot conclude (as the authors do) that Smurf1 and 2 are specifically FBXL15 substrates, because surprisingly the authors chose Nedd4-1 (which binds only very weakly) and AIP4 (intermediate binding), but they did not the test with NEDL1 and 2, which clearly bind the strongest to FBXL15. In view of the findings on binding in 5A, they have to test all the enzymes in order to draw any conclusions, and they should as well do the ubiquitination experiments in Hek293 cells.

Fig 6 D: The quantification of the immunoblots is missing.

Fig 7: In this part the authors wanted to determine the effects of FBXL15 on TGF-beta and BMP signaling pathway. Using exclusively some luciferase reporter gene, the authors conclude that FBXL15 enhances the BMP and TGF-beta induced transcription. Unfortunately, a deep work on the function of FBXL15 in this signaling pathway is missing, e.g.: Different studies demonstrated that Smurf1 and Smurf2 target TGF-beta receptor for degradation, one experiment could be to check the phosphorylation level of the downstream Smads in presence or absence of FBXL15.
- The effects of FBXL15 on endogenous genes induced by TGF-beta or BMP
- The effect of FBXL15 on cell response (such as proliferation)

Minor issues:
- The authors conducted all their experiments in cells. The "in vivo" expression is used when the experiments were done in a whole living organism. The term "endogenously" is preferable.
- Page 4 Space is missing between words "type I receptor"
- The frame surrounding certain immunoblot is missing, and the thickness of the line is not the same for all the immunoblots.

Referee #2:

In this paper, the authors describe the identification of the SCF protein FBXL15 as a binding partner for Smurf1 (and Smurf2), an interaction that leads to ubiquitination and degradation of Smurf1 by the SCF-FBXL15 complex, thus attenuating BMP and TGFbeta signaling. FBXL15 binds via its LRR the N-terminal lobe of the Smurf1-Hect domain, a novel interaction. As stated by the authors, this is the first case reported of an SCF E3 ligase regulating a stability of a Hect ligase.

Overall, this is an interesting paper. Several papers have been published over the past few years, which document negative regulation of Hect E3s function by intra/inter-molecular interactions (eg C2 or WW domains binding to the Hect domain) or by phosphorylation, as well as by adapter proteins. In addition, Smurf proteins were previously shown to interact with a RING ligase, Rnf11. The present paper is unique, though, in that it documents regulation of stability of Smurf E3s by an SCF complex, leading to Smurf degradation and enhanced BMP signaling. What this work lacks is a mechanistic explanation as to what physiologically stimulates (ie signals) FBXL15 to interact with and regulate stability of the Smurfs.

Specific points:
1. Why was the E2 UbcH5c used in the reactions to study Smurf ubiquitination? What happens when UbcH7 is used? (Previous structural and functional work demonstrated a role for UbcH7 in binding to Smurf2 and regulating its function).
2. In Fig 2E, if Smurf1 can become ubiquitinated presumably (as proposed by the authors) by endogenous SCF-FBXL15 (lane 2), why don't you see ubiquitination of catalytically-inactive Smurf1-CA (lane 7)?
3. In Fig 5C, a lower exposure of the ubiquitin blot for Nedd4-1 is needed, as it appears that
FBXL15 actually increases Nedd4-1 ubiquitination (which does not fit with the hypothesis of the authors, since Nedd4-1 does not possess K357).

4. In Fig 6D, the data are not very convincing and the results lack quantification and statistics.

5. In Fig 7B, how is the F box region alone able to increase BRE activity? (ie compare lane 7 to lane 4)

6. In Fig 7D, the authors should include the triple K to R mutant in the analysis, since even the strongest mutant (K357R) was not able to fully restore BRE activity.

7. It appears that only a single siRNA was used, raising the possibility of off-targets effects. This experiments should be performed with several siRNAs (or at least with a rescue of the siRNA, ie with FBXL15 re-introduction into cells).

8. The authors raised antibodies to FBXL15, but their characterization is not shown. In particular, in Suppl Fig S1(C) only a slice of a blot is shown with no molecular wt markers. The authors should properly characterize the antibodies (with positive and negative controls), and show a full size blot, to ensure specificity of the antibodies.

9. The description of the ubiquitination (in cells) experiments is not clear (page 27): cells were lysated in RIPA-modified buffer. Was the lysate boiled and was it diluted prior to the IP?

10. The paper is fraught with many grammatical errors to the point that it is sometimes difficult to understand what the authors intend to say. In addition, several sentences/paragraphs are simply incomplete (eg page 26, first line of the section" Immunoprecipitation and immunoblotting", and the legend to Fig 6E, which anyway claims attenuation of ubiquitination of Smurf2, opposite of what the figure actually shows).

11. In general, given that there have been quite a few papers published to date describing the regulation of function of Nedd4 members, it is probably a good idea to tone down the claims of lack of such knowledge (in the Introduction and Discussion).

Referee #3:

All proteins that reside in the cytoplasm are subject to turnover, the vast majority probably through the action of the ubiquitin-proteasome system. It is therefore hardly surprising that proteins involved in Ub conjugation are themselves targeted in this fashion, and have their levels controlled through ubiquitin-dependent proteasomal proteolysis. One way of looking at this work, therefore, would be to draw the conclusion that a cytoplasmic protein turns over under the agency of the Ub-proteasome system, hardly a startling conclusion.

The authors provide a reasonable analysis of the fate of Smurf1, a HECT domain E3 ligase, and implicate FBXL15 in the process. I see no particularly novel elements in this manuscript, nor does it materially affect our understanding of the Ub-proteasome pathway in general, or of the mode of action of HECT domain E3 ligases in particular. I see no new insights in our understanding of TGF signaling that would merit publication of this work in EMBO J. It may well be true that no previous link has been established between RING domain Ub ligases and their regulation of HECT domain-containing E3's, but as presented, this material would be much better placed in a journal such as FASEB J., Biochem. J. or maybe J. Biol. Chem.

The identification of FBXL15 in a two hybrid screen by no means excludes a similar role for other FBXL family members, and the very minimal survey of other SCF-type ligases cannot even remotely be construed as evidence for specificity.

A main drawback of this work, as is true for many related studies, is the heavy reliance on overexpressed, epitope tagged constructs, with almost no attention paid to the fate of proteins expressed at endogenous levels (exception:fig 2A). This would require a different experimental approach, such as the generation of antibodies that recognize the native products in untagged form, and preferably the use of metabolic labeling methods and accurate quantitation, instead of the exclusive use on immunoblots, GST pulldowns and the like. The exposures of some of the blots are chosen to maximize differences (fig 1), many of poor quality (1B,F,G;2E; the poly Ub blots). In the absence of any information on the dynamic range of such measurements, I have little confidence in the quantitations, where the authors simply equate loss of an epitope (no longer detectable by immunoblots) with complete loss of the protein(s) in question. The conclusion drawn from fig 1G is
simply not sustained by the quality of the figure shown, and undercuts much of the author's conclusions. The immunoprecipitation/immunoblotting protocols should ideally be performed on samples of which the composition can be assessed more accurately: evidence for direct Ub conjugation (mass spec) is absent. For the key experiment in fig. 7 (the only one that addresses some aspects of function in a rather contrived system) absolute values, rather than fold increases should be given. The data in panel A show disconcerting variation in the levels of Smurf1 and FBXL15 when assessed relative to each other. These experiments should include other members of the FBXL family as well.

Papers that deal with the Ub-proteasome system increasingly show a tendency to extremely formulaic introductions: almost all of them read the same, and those in the field could skip page 1 without missing relevant information. For the TGF experts, the same is true for p. 2, and this part can be omitted almost entirely since the subject of TGF signaling itself hardly makes an appearance in the experimental section (exception fig 7). The text should be checked for proper idiomatic usage (agreement of subject and verb; dropped articles, superfluous articles, etc etc) of the English language by a native speaker.

Additional Author Correspondence
10 March 2010

Thanks for your email. We have read the referees' comments carefully. Although the current manuscript does not meet the standard for publication in your journal, we found that the referees appreciated the whole story and the major findings in this study. We agree with the referees' comments and we are grateful to them for these suggestions and criticisms. Most of the questions can be answered and we decided to amend the experiments according to the referees' suggestions. When we finished the experiments, improved our manuscript, we will resubmit the manuscript to your excellent journal.

Resubmission
27 January 2011

This is a revised version of our previous manuscript EMBOJ-2010-73948. Many thanks for your kind consideration of this manuscript. All of the comments, criticisms and suggestions from you and the three referees have been very useful for improving the manuscript. We have now finished all of the related experiments, reorganized the figures, revised the manuscript and addressed all of the reviewers' questions. They are listed below point-by-point. We hope this revised manuscript will be considered for publication in your excellent journal.

Responses to the Editor:
Comments: Thank you for submitting your research manuscript to The EMBO Journal editorial office. The work has been seen by three expert referees who's assessment you will find enclosed below for your information. As you will see, refs#1 and #2 appreciate some interest in your findings whereas ref#3 does find the conceptual insight rather limited. Careful reading of all the reports reveals that apart from this conceptual concern, the experimental evidence and in particular physiological significance of the proposed regulation of Smurf1 by FBXL15 remains currently uncertain. As this is a rather important measure at least from the perspective and scope of our more general and very competitive journal, I am very sorry to have to communicate that we all had to conclude that we are unable to offer any further proceedings at this preliminary state of analyses.

Response (R): We received the reviewers' comments in March 2010. During the last ten months, we have performed many experiments according to the reviewers' suggestions and yours. In the resubmitted manuscript, we have provided strong evidence to indicate that FBXL15 organizes an intact SCF-type ubiquitin ligase to promote the ubiquitination and proteasomal degradation of Smurf1. More importantly, we show that FBXL15 plays a critical role in the regulation of BMP signaling in vivo, i.e., both in embryonic development (with RNAi in zebrafish embryos as the model) and in adult bone remodeling (with RNAi in rat bones as the model). As far as we know, this is the first case to report an F-box protein’s role in the determination of the
dorsalization/ventralization axis and in bone homeostasis control. In addition, the quality of the data has been significantly improved, and many results based on endogenous experiments are provided. We hope the modified manuscript will meet the standard for publication in the EMBO Journal.

Responses to Reviewer #1:

Comments: In this paper, Cui et al.; present a series of experiments that address the potential role of FBXL15 in the degradation of Smurf1 and Smurf2 through the SCF<sup>FBXL15</sup> mediated complex. This is a novel study demonstrating that turn-over of a HECT type E3 enzyme could be regulated in trans by a SCF complex. While the findings are of potential interest in HECT E3 regulation, there are several concerns I have regarding the experiments and the data interpretation.

R: Many thanks for your kind support regarding the novelty of our manuscript. In view of your concerns, we have performed a number of experiments and provided new data that are described below point-by-point.

**Question 1 (Q1):** Fig 1 B: The authors demonstrate that FBXL15 belongs to the SCF complex composed by Cullin1, Skp1 and Roc1. To prove this hypothesis, the authors over-expressed these different proteins and show that they can be pulled down by GST-FBXL15 wt, but not with the delta-F mutant. To be confident with the kind of SCF complex we are dealing with, this observation needs to be confirmed by co-immunoprecipitation, ideally with endogenous proteins.

R: More convincing data have been provided. According to your suggestion, co-immunoprecipitation assays in human cells were performed. Fig. 2A of the revised manuscript shows that Flag-tagged full-length FBXL15, but not ΔF mutant, was co-immunoprecipitated with endogenous Cullin 1, Skp1 and Roc1. We attempted to co-purify the endogenous FBXL15 complex with Cullin 1, Skp1 and Roc1, but failed due to the insufficient efficiency of the anti-FBXL15 antibody in the immunoprecipitation assay. Nonetheless, the semi-endogenous co-immunoprecipitation assay data indicate that FBXL15 does organize an intact SCF ligase complex in mammalian cells.

**Q2:** Fig 1 C: The authors show that over-expression of FBXL15 down-regulates the expression of Smurf1 wt and also the Smurf1 CA mutant. They conclude that FBXL15 induced degradation is beyond Smurf1 activity. However, on the western-blot, we clearly see that Smurf1 CA mutant is less down regulated than the wild-type by FBXL15. The authors should quantify the blots and provide some statistics. If the inactive mutant is less suppressed, it will be necessary to provide some explanations. For example they should test if the CA mutant is less well interacting with FBXL15 or is less ubiquitylated. Or they may want to carry out a dose response of FBXL15.

R: According to your suggestion, we repeated the experiment with a dose increase of FBXL15 and detected the expression levels of Smurf1 WT and C699A more carefully. As shown in revised Fig. 2B, the WT and CA forms of Smurf1 were down regulated by FBXL15 in a dose-dependent manner and to a similar extent (please see the quantitative analysis in Fig. 2B, bottom). Treatment with proteasome inhibitor MG132 largely prevented this down regulation. Because we also showed that the N-lobe large subdomain of Smurf1 HECT N-lobe mediates the interaction with FBXL15 (note that the catalytic residue C699 is located within the C-lobe) (Fig. 1), it is reasonable that the C699A mutation had no significant effect on the interaction with FBXL15 or on the degradation effect.

**Q3:** Fig 1G is not very convincing. Please provide quantification and statistics.

R: Better data have been included in the revised version (Fig. 2D). Two independent FBXL15-targeting siRNAs were used in this experiment. Depletion of FBXL15 resulted in a significant increase in the endogenous Smurf1 protein level. Quantification data are also shown.

**Q4:** Fig 2A: The authors have to show the immunoblot of FBXL15 to evaluate the efficiency of FBXL15 silencing in this experiment.

R: Better data have been included in the revised version (Fig. 2E). Two independent FBXL15-targeting siRNAs were used to test the effect of FBXL15 on endogenous Smurf1. As shown, the half-life of Smurf1 is significantly prolonged in the depletion of endogenous FBXL15. The high efficiency of the two FBXL15 siRNAs was shown in Fig. 2D.
Q5: Fig 2A and 2B: The half life of Smurf1 alone is approximately 11 hours (Fig 2B). When Smurf1 is co-transfected with a control siRNA which is supposed to not disturb the protein expression; the half life of the protein falls to approximately 6 hours (Fig 2A). This discrepancy is surprising because the addition of FBXL15 siRNA restores the half-life of Smurf1 to 12 hours which is approximately the same as Smurf1 alone (Fig 2B).

R: We regret the inconsistency of the results, which was probably caused by the deviations among different experiments. During preparation of the revised manuscript, we carefully repeated many experiments for which we had low-quality data in the previous version and improved the data quality. As shown in Fig. 2E, the half-life of endogenous Smurf1 is approximately 6 hours in the control cells and is prolonged to more than 9 hours in the FBXL15-depleted cells. As shown in Fig. 3G, the half-life of exogenous Smurf1 is also approximately 6 hours in the control cells and is shortened to 4 hours in the FBXL15-overexpressed cells.

Q6: Fig 4A: Using different fragments of Smurf1, the authors find that the region 234-372 contains the signal for FBXL15 degradation. However, the study is not complete; they have to provide us also with the fragment aa 1 to 234 corresponding to the C2 domain.

R: To address this issue, we constructed a Smurf1 mutant (ΔWHL) lacking the WW-HECT linker (aa 313-372). As shown in Fig. 3D, the ΔWHL mutant was resistant against the FBXL15-promoted degradation (lanes 10-12), in contrast to the effect of full-length Smurf1 (lanes 1-3) and WH truncation (lanes 4-6). This result suggests that the C2 domain is not sufficient to mediate the degradation effect of FBXL15.

Q7: Fig 4 C and D: The authors mention in the text that Smurf1 mutant K357R is "completely resistant to the degradation by FBXL15". It is clear that this mutation decreased the effect of FBXL15 on Smurf1, however this decreases seems to be not totally blocked. To give further evidence for this hypothesis, the authors have to assess the half-life of Smurf1, Smurf1 K357R, and finally with Smurf K355+357R + FBXL15. The blots have to be quantified.

R: As described above, we improved the data quality of many figures to make the conclusions more convincing. The revised figure 3G shows that the protein levels of the Smurf-K355+357R double mutant in the absence of exogenous FBXL15 were similar to those in the presence of exogenous FBXL15 (lanes 13-16 versus 9-12, and the right degradation curve), indicating that the K355+357R mutation conferred resistance against FBXL15-mediated degradation.

Q8: Fig. 4E. In vitro ubiquitinations are not very specific. The authors should test the ubiquitination of Smurf1 in Hek293 cells, as they have done in Fig. 2C.

R: The former version of Fig. 4E showed only the in vivo ubiquitination assay in HEK293 cells. In the revised Fig. 3H, we show that the K355+357R mutation prevents the FBXL15-promoted poly-ubiquitination of Smurf1 in Hek293 cells.

Q9: Fig 5: In this figure the authors show that Smurf1 and 2, but not the other Nedd4 related enzymes are a target of FBXL15. They do that by co-ips, in which they find that all the Nedd4 related enzymes interact with FBXL15. Interestingly there are differences, as Smurf1 and 2, but even more so NEDL1, NEDL2, bind stronger, and Nedd4-1 and Nedd4-2 show weaker binding. Then they test the effect of FBXL15 on stability of 4 of these enzymes (Smurf1 and 2; Nedd4-1 and AIP4), and find that only Smurf1 and 2 are affected. With the same enzymes they carry out in vitro ubiquitination assays. However, from these experiments one cannot conclude (as the authors do) that Smurf1 and 2 are specifically FBXL15 substrates, because surprisingly the authors chose Nedd4-1 (which binds only very weakly) and AIP4 (intermediate binding), but they did not test with NEDL1 and 2, which clearly bind the strongest to FBXL15. In view of the findings on binding in 5A, they have to test all the enzymes in order to draw any conclusions, and they should as well do the ubiquitination experiments in Hek293 cells.

R: Thank you for the useful advice. In view of this comment, we performed protein-protein interaction, protein degradation and protein ubiquitination assays, and we show the data in revised
Fig. 5A, 5B, 5C, respectively. Fig. 5A shows that under conditions of overexpression, FBXL15 can be co-immunoprecipitated with each of the Nedd4 family ligases. This result is not surprising, as we proved that the N-lobe large subdomain of Smurf1 mediates the interaction with FBXL15 (Fig. 1H, 1I), and this region is conserved among the nine family members. The difference in binding affinity might be caused by the sequence differences among them, as they are homologous but not identical. Fig. 5B shows that Smurf1, Smurf2 and WWP2, but not the other six members, were downregulated by FBXL15, indicating the specificity. Fig. 5C shows that the poly-ubiquitination of Smurf1 and Smurf2 was significantly promoted by FBXL15. By contrast, the ubiquitination of Nedd4-1, NEDL2, WWP1 and WWP2 was less promoted or not significantly promoted by FBXL15. A possible explanation for this phenomenon may be the difference of the ubiquitination site—lysine. Supplementary Figure S4 shows that the corresponding residue of Smurf1-K357 in other E3s is only conserved in Smurf2. At present, we do not know the exact mechanism by which WWP2 is also degraded by FBXL15, which should be further investigated in the future. Collectively, these data suggest that FBXL15 targets Smurf1 and Smurf2 for ubiquitination and degradation with a relatively high specificity, at least among the Nedd4 family of E3 ligases.

Q10: Fig 6 D: The quantification of the immunoblots is missing.
R: These data no longer appear in the revised manuscript.

Q11: Fig 7: In this part the authors wanted to determine the effects of FBXL15 on TGF-beta and BMP signaling pathway. Using exclusively some luciferase reporter gene, the authors conclude that FBXL15 enhances the BMP and TGF-beta induced transcription. Unfortunately, a deep work on the function of FBXL15 in this signaling pathway is missing, e.g.:  
- Different studies demonstrated that Smurf1 and Smurf2 target TGF-beta receptor for degradation, one experiment could be to check the phosphorylation level of the downstream Smads in presence or absence of FBXL15.
- The effects of FBXL15 on endogenous genes induced by TGF-beta or BMP
- The effect of FBXL15 on cell response (such as proliferation)
R: The reviewer’s suggestions have been very useful for improving our manuscript. We have added new and critical data to address these questions.

First, as shown in Fig. 4B, depletion of FBXL15 by RNAi resulted in a significant attenuation of BMP-2-induced Smad1/5 phosphorylation, which is triggered by the BMP receptor at the C-terminus of Smad1/5, indicating a positive role of FBXL15 in BMP signal transduction.

Second, depletion of FBXL15 by RNAi resulted in a significant decrease in the expression of Smad1/5 target genes, including ID1 and Smad6 (Fig. 4F), and attenuation of BMP response element (BRE) luciferase activity (Fig. 4E).

Third, the role of FBXL15 in embryonic development was investigated using zebrafish as a model. As shown in Fig. 6, depletion of FBXL15 in zebrafish embryos through injection of anti-FBXL15 morpholinos resulted in a significant dorsalization phenotype and aberrant expression of BMP-Smad downstream targets, which are similar to the phenotypes of Smurf1 ectopic expression in Xenopus embryos (Zhu et al., 1999). Ectopic expression of FBXL15 in the zebrafish embryos dramatically inhibited the expression of Smurf1/2 protein.

Fourth, we asked whether FBXL15 also plays a role in BMP signaling during adult development, especially in the maintenance of bone homeostasis. Smurf1 has been determined to specifically suppress bone formation in mammals (Yamashita et al., 2005; Zhao et al., 2003; Zhao et al., 2010). We then hypothesized that FBXL15, as a negative regulator of Smurf1, plays a positive role in bone formation. If true, FBXL15 depletion should lead to a decrease in bone mass. Indeed, depletion of FBXL15 in six-month rats significantly decreased the apparent BMD, relative bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb. N). Importantly, less organized three-dimensional architecture and lower bone mass in trabecular bone were clearly found in rats treated with the FBXL15 siRNA compared to the control groups (Fig. 7A-7G). Collectively, these data strongly indicate that FBXL15 is a positive regulator of bone mass maintenance in adult mammals.

Collectively, these results suggest that FBXL15 plays a positive role in BMP signaling control.
Minor issues:

Q12: The authors conducted all their experiments in cells. The "in vivo" expression is used when the experiments were done in a whole living organism. The term "endogenously" is preferable.

R: We thank the reviewer for this comment. We have corrected these descriptions in the revised manuscript. For example, page 7, description of Fig. 1, “Together these data demonstrate FBXL15 interacts with Smurf1 both in vitro and endogenously in cultured cells.” Page 16, description Fig. S5, “GST pull-down assay (Supplementary Figure S5A) and endogenous co-immunoprecipitation experiments (Supplementary Figure S5B) indicated that Smurf2 could interact with FBXL15 both in vitro and endogenously in cultured cells, similar to Smurf1.” Also, legend of Figure 1, “Smurf1 interacts with FBXL15 both in vitro and in cultured cells”.

Q13: Page 4 Space is missing between words "type I receptor"

R: This sentence no longer appears in the revised manuscript.

Q14: The frame surrounding certain immunoblot is missing, and the thickness of the line is not the same for all the immunoblots.

R: We have checked and adjusted all of the frames surrounding the immunoblots to make the format consistent.

Responses to Reviewer #2:

Comments: In this paper, the authors describe the identification of the SCF protein FBXL15 as a binding partner for Smurf1 (and Smurf2), an interaction that leads to ubiquitination and degradation of Smurf1 by the SCF-FBXL15 complex, thus attenuating BMP and TGFbeta signaling. FBXL15 binds via its LRR the N-terminal lobe of the Smurf1-Hect domain, a novel interaction. As stated by the authors, this is the first case reported of an SCF E3 ligase regulating a stability of a Hect ligase.

Overall, this is an interesting paper. Several papers have been published over the past few years, which document negative regulation of Hect E3s function by intra/inter-molecular interactions (e.g. C2 or WW domains binding to the Hect domain) or by phosphorylation, as well as by adapter proteins. In addition, Smurf proteins were previously shown to interact with a RING ligase, Rnf11. The present paper is unique, though, in that it documents regulation of stability of Smurf E3s by an SCF complex, leading to Smurf degradation and enhanced BMP signaling. What this work lacks is a mechanistic explanation as to what physiologically stimulates (i.e. signals) FBXL15 to interact with and regulate stability of the Smurfs.

R: Many thanks for the reviewer’s appreciation and support of our manuscript, including the novelty. In the revised manuscript, we added genetic evidence to show that FBXL15 plays a critical role in embryonic development and adult bone homeostasis. As indicated in Fig. 6, depletion of FBXL15 in zebrafish embryos resulted in a significant dorsalization phenotype and an aberrant expression of BMP-Smad downstream targets. As indicated in Fig. 7, depletion of FBXL15 in six-month rats significantly decreased the apparent BMD, relative bone volume, trabecular thickness and trabecular number. Less organized three-dimensional architecture and lower bone mass in trabecular bone were clearly found in the rats treated with the FBXL15 siRNA. Additionally, depletion of FBXL15 in human cells also resulted in a significant decrease in BMP or TGF-β signaling (Fig. 4 and 5). Therefore, these data support the notion that FBXL15 is a new positive regulator of BMP/TGF-β signaling in human, rat and zebrafish. We hope these modifications adequately address the reviewers concerns.

Q1: Why was the E2 UbcH5c used in the reactions to study Smurf ubiquitination? What happens when UbcH7 is used? (Previous structural and functional work demonstrated a role for UbcH7 in binding to Smurf2 and regulating its function).
R: Both UbcH5c and UbcH7 were tested for their roles in FBXL15-mediated Smurf ubiquitination. As shown in Fig. 3B, either UbcH5c or UbcH7 can coordinate with FBXL15 to promote the poly-ubiquitination of Smurf1, indicating the similar roles of UbcH5c and UbcH7 in this reaction.

Q2: In Fig 2E, if Smurf1 can become ubiquitinated presumably (as proposed by the authors) by endogenous SCF-FBXL15 (lane 2), why do not you see ubiquitination of catalytically-inactive Smurf1-CA (lane 7)?

R: We think our explanation was unclear and led to a misunderstanding of this result. Lane 2 shows the auto-ubiquitination of His-Smurfl in the presence of E1/E2/ATP and HA-Ub (all of which are purified in bacteria), whereas lane 7 indicates that Smurf1-C699A lost the ability of auto-ubiquitination. Note that, in these two groups, no endogenous SCF-FBXL15 or cell extracts were included in the reaction. Thus, we cannot observe the ubiquitination of Smurf1 (WT or CA) by basal SCF-FBXL15. When exogenous SCF-FBXL15 ligase complex (purified from mammalian cells) was added to the reaction, both Smurf1-WT and CA ubiquitination were significantly enhanced (lanes 3 versus 2 and 8 versus 7, respectively), suggesting that SCF-FBXL15 promotes Smurf1 ubiquitination independent of the Smurf1 ligase activity. However, this process required the entire SCF complex, as SCF-FBXL15-delta-F (lane 4) or GST-FBXL15 alone (lack Cullin1, Skp1 and Roc1) cannot efficiently promote the Smurf1 ubiquitination (Fig. 3C).

Q3: In Fig 5C, a lower exposure of the ubiquitin blot for Nedd4-1 is needed, as it appears that FBXL15 actually increases Nedd4-1 ubiquitination (which does not fit with the hypothesis of the authors because Nedd4-1 does not possess K357).

R: This experiment has been repeated, and more E3 ligases were included. As clearly shown in revised Fig. 5C, the ubiquitination of Smurf1 and Smurf2 were significantly enhanced by FBXL15; by contrast, the ubiquitination of Nedd4-1, NEDL2, AIP4 and WWP2 was not enhanced or was less significantly enhanced by FBXL15. These data suggest that the poly-ubiquitination of Smurf1 and Smurf2, among the Nedd4 family of E3 ligases, is specifically promoted by FBXL15.

Q4: In Fig 6D, the data are not very convincing and the results lack quantification and statistics.

R: In the revised manuscript, this figure no longer appears.

Q5: In Fig 7B, how is the F box region alone able to increase BRE activity? (i.e. compare lane 7 to lane 4)

R: We regret the inaccurate data, which might have been caused by deviation in the experimental operations. These experiments have been repeated carefully, and better data are provided in the revised Fig. 4C. As shown, the F-box of FBXL15 alone had no significant effect on Smurf1-mediated BRE inhibition (column 7 versus 4), suggesting that a direct interaction between Smurf1 and FBXL15 is required for FBXL15 to regulate Smurf1 activity.

Q6: In Fig 7D, the authors should include the triple K to R mutant in the analysis because even the strongest mutant (K357R) was not able to fully restore BRE activity.

R: Thank you for your advice. As K324 is not the ubiquitination site for FBXL15, we constructed a double mutant K355+357R. As shown in Fig. 4D, wild-type Smurf1 and all of the examined Smurf1 mutants (including K324R, K355R, K357R) efficiently inhibited the BMP-triggered BRE activity in the absence of ectopic FBXL15, suggesting that these mutations had no effects on the ligase activity of Smurf1 itself. When FBXL15 was co-expressed, the inhibitory effect of Smurf1 WT, K324R and K355R was completely reversed. By contrast, the double mutant K355+357R was resistant against FBXL15, whereas K357R was partially resistant against FBXL15. These data strongly indicate that FBXL15 regulates Smurf1 activity on BRE transactivation in a manner dependent on the ubiquitination of K355 and K357.
Q7: It appears that only a single siRNA was used, raising the possibility of off-target effects. This experiment should be performed with several siRNAs (or at least with a rescue of the siRNA, i.e. with FBXL15 re-introduction into cells).

R: Thank you for the suggestion. In the revised Fig. 2D, 2E, 4E, 4F and 5F, two independent siRNAs that both efficiently knock down FBXL15 were used in the experiments, and both results are shown. Both siRNAs showed similar results and thus made the conclusion more convincing, as the reviewer recommended. Furthermore, depletion assays in zebrafish embryos and rats were performed and are shown in Fig. 6 and Fig. 7.

Q8: The authors raised antibodies to FBXL15, but their characterization is not shown. In particular, in Suppl Fig S1(C)) only a slice of a blot is shown with no molecular wt markers. The authors should properly characterize the antibodies (with positive and negative controls), and show a full size blot, to ensure specificity of the antibodies.

R: As shown in revised Supplementary Fig S1B-S1D, the anti-FBXL15 antibody recognizes both endogenous and exogenous FBXL15 proteins with a molecular weight of 38 kDa (Fig. S1B), and the antibody-recognizing band was significantly reduced in the FBXL15 siRNA-transfected cells (Fig. S1C), further confirming the specificity of this antibody. The FBXL15 siRNA had no significant effects on the expression of other F-box proteins (Fig. S1D). We also utilized this antibody to examine the expression profile of FBXL15 in various tumor cell lines and mouse organs, and we showed that FBXL15 is widely expressed in the examined samples (Fig. S1E-S1F).

Q9: The description of the ubiquitination (in cells) experiments is not clear (page 27): cells were lysated in RIPA-modified buffer. Was the lysate boiled and was it diluted prior to the IP?

R: The detailed experimental procedures have been described in the revised manuscript (pages 28-29).

Q10: The paper is fraught with many grammatical errors to the point that it is sometimes difficult to understand what the authors intend to say. In addition, several sentences/paragraphs are simply incomplete (eg page 26, first line of the section "Immunoprecipitation and immunoblotting", and the legend to Fig 6E, which anyway claims attenuation of ubiquitination of Smurf2, opposite of what the figure actually shows).

R: We apologize for the grammatical errors in the manuscript. The current revised manuscript has been edited by an expert company, American Journal Experts (http://www.journalexperts.com), and we therefore believe the grammatical errors have been avoided.

Q11: In general, given that there have been quite a few papers published to date describing the regulation of function of Nedd4 members, it is probably a good idea to tone down the claims of lack of such knowledge (in the Introduction and Discussion).

R: The reviewer’s suggestion is reasonable. We have toned down these claims in the revised manuscript.

Response to Reviewer #3:

Comment 1: All proteins that reside in the cytoplasm are subject to turnover, the vast majority probably through the action of the ubiquitin-proteasome system. It is therefore hardly surprising that proteins involved in Ub conjugation are themselves targeted in this fashion, and have their levels controlled through ubiquitin-dependent proteosomal proteolysis. One way of looking at this work, therefore, would be to draw the conclusion that a cytoplasmic protein turns over under the agency of the Ub-proteasome system, hardly a startling conclusion.
The authors provide a reasonable analysis of the fate of Smurf1, a HECT domain E3 ligase, and implicate FBXL15 in the process. I see no particularly novel elements in this manuscript, nor does it materially affect our understanding of the Ub-proteasome pathway in general, or of the mode of action of HECT domain E3 ligases in particular. I see no new insights in our understanding of TGF signaling that would merit publication of this work in EMBO J. It may well be true that no previous link has been established between RING domain Ub ligases and their regulation of HECT domain-containing E3’s, but as presented, this material would be much better placed in a journal such as FASEB J., Biochem. J. or maybe J. Biol. Chem.

R: Thank you for these criticisms. This reviewer previously thought this manuscript lacked sufficient novelty for publication in EMBO Journal. We have carefully read all of the three reviewers’ comments and inquiries regarding our work and have performed many experiments to improve the manuscript according to these valuable suggestions. Both the quality of the data and the quantity of the figures are significantly improved. We believe that the REVISED manuscript may be of interest to the broad readership that the EMBO Journal enjoys. We believe the paper will appeal to readers from different backgrounds. The “protein ubiquitination”, “SCF E3 ligase”, “HECT-type E3 ligase”, “embryonic development” and “bone remodeling” audiences should all find some interest in the work, for which we brought together very diverse techniques and areas of expertise.

There are at least three lines of novelty in our work:

First, Smurf1 has recently received considerable scientific attention for being a major regulator of embryonic development, bone remodeling and cell polarity control. Smurf1 was originally identified as the first E3 ubiquitin ligase of Smad1/5, the signal transducer of the BMP pathway, and it proved to play a critical role in regulation of frog embryogenesis (Zhu et al., Nature, 1999). Subsequently, Smurf1 was determined to target RhoA (Wang et al., Science, 2003), MEKK2 (Yamashita et al., Cell, 2005) and Prickle 1 (Narimatsu et al., Cell, 2009) for ubiquitination and proteasomal degradation, thereby regulating bone formation and embryonic development. Such a critical regulator should be tightly controlled, both positively and negatively. For the activation mechanism, we recently showed that CKIP-1 functions as the first identified auxiliary factor of Smurf1 and promotes the role of Smurf1 in bone formation regulation (Lu et al., Nature Cell Biology, 2008). At present, the INHIBITION mechanism of Smurf1 remains largely unclear. In this manuscript, we established that the SCF^FBXL15 ubiquitin ligase complex targets Smurf1 for degradation. This regulation should be important to maintain the homeostasis of Smurf1 stability under healthy conditions.

Second, this is the first evidence to show HECT domain type ubiquitin ligase to be a substrate of an SCF type ubiquitin ligase. Although most of the ubiquitin ligases can catalyze auto-ubiquitination and then self-degradation, this ability is not sufficient for efficient control of E3 ligase activity under certain spatial-temporal conditions. The current work showed that an HECT E3 can be targeted by an SCF E3, which represents the best-characterized RING finger type E3 ligases. We precisely mapped the large subdomain of Smurf1 HECT N-lobe as the interacting site with FBXL15, and the K355 and K357 residues are the ubiquitination sites for FBXL15. As far as we know, this is the first study to precisely map the ubiquitination site on HECT E3s. Previous studies have identified that RING E3s can be the substrate of HECT E3s; for example, c-Cbl and RNF11 are the substrates of Nedd4-1 (Magnifico et al., 2003, JBC) and Smurf2 (Subramaniam et al., Br J Cancer, 2003), respectively. A recent study demonstrated that the N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases (Hwang et al., Nature Cell Biology, 2010). Thus, the concept of “ubiquitinating the ubiquitinase” might exist in more cases.

Third, this is the first evidence showing a role of F-box protein in the regulation of dorsalization, ventralization and bone homeostasis. There are 69 human F-box proteins, but the majority of them remain uncharacterized. After a careful systematic analysis of known F-box proteins, we found that seven FBXL members, four FBXW members and eleven FBXO members have previously been matched with substrates (Skaar et al., Cell, 2009, 137:1358.e1. SnapShot: F Box Proteins II.). In another word, nearly 70% of the F-box proteins remain functionally unclear. Among the demonstrated F-box proteins, none had been shown to play any role in the determination of the

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dorsal/ventral axis or bone homeostasis control. In this regard, we think our findings will be of interest to the readers. We provide strong evidence to show the depletion of FBXL15 in zebrafish embryos (Fig. 6) and in rat bone tissues (Fig. 7), resulting in significant phenotypes.

Comment 2: The identification of FBXL15 in a two hybrid screen by no means excludes a similar role for other FBXL family members, and the very minimal survey of other SCF-type ligases cannot even remotely be construed as evidence for specificity.

R: The previous manuscript used Skp2 (also called FBXL1), β-TrCP1 (also called FBXW1) and FBXO42 (also called JFK) as the controls. This number is indeed limited. In the revised Fig. 2F, three more FBXL subfamily members are included in the degradation assays. As shown, in contrast to FBXL15, neither FBXL3, FBXL5, FBXL21 nor Skp2, β-TrCP1 and FBXO42 had any significant effects on Smurf1 protein level, indicating the specificity of FBXL15. We think the total of six controls should be sufficient to explain the specificity. Similar experiments can be found in three prior references. One is a Science paper in 2007, which showed that FBXL3 controls the oscillation of the circadian clock by targeting cryptochrome proteins for degradation. The authors compared FBXL3 with β-Trcp1, Skp2, FBXL2, FBXL10, FBXL11, FBXL15, Emi1, FBXO9 and FBXO22 (Busino et al., SCF\textsuperscript{null} controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. Science 2007, 316: 900). The second case is a Nature Cell Biology paper in 2007, which showed that FBXL10 interacts with c-Jun and represses c-Jun-mediated transcription. The authors compared FBXL10 with FBXL15, FBXL12, FBXL7, FBXL2 and Skp2 (Koyama-Nasu et al., The F-box protein Fbl10 is a novel transcriptional repressor of c-Jun. Nature Cell Biology 2007, 9: 1074). The third case is an Oncogene paper in 2009, which showed that FBXO45 promotes the proteasome-dependent degradation of p73. The authors compared FBXO45 with β-TrCP1, FBXW7, FBXO4, FBXO11 and FBXO28 (Peschiaroli et al., The F-box protein FBXO45 promotes the proteasome-dependent degradation of p73. Oncogene 2009, 28: 3157).

Notably, FBXL15 was used in the former two references as a control F-box protein and displayed no effects on the substrates of FBXL3 or FBXL10, further confirming the substrate specificity of different F-box proteins.

Comment 3: A main drawback of this work, as is true for many related studies, is the heavy reliance on overexpressed, epitope tagged constructs, with almost no attention paid to the fate of proteins expressed at endogenous levels (exception: fig 2A). This would require a different experimental approach, such as the generation of antibodies that recognize the native products in untagged form, and preferably the use of metabolic labeling methods and accurate quantitation, instead of the exclusive use on immunoblots, GST pull downs and the like. The exposures of some of the blots are chosen to maximize differences (fig 1), many of poor quality (1B,F,G;2E; the poly Ub blots). In the absence of any information on the dynamic range of such measurements, I have little confidence in the quantitations, where the authors simply equate loss of an epitope (no longer detectable by immunoblots) with complete loss of the protein(s) in question. The conclusion drawn from fig 1G is simply not sustained by the quality of the figure shown, and undercuts much of the author’s conclusions. The immunoprecipitation/immunoblotting protocols should ideally be performed on samples of which the composition can be assessed more accurately: evidence for direct Ub conjugation (mass spec) is absent.

R: These suggestions are excellent and were very useful for improving our manuscript. In the revised manuscript, more endogenous data are included. For example, Fig. 1C, 2D, 2E, 2F, 4A, 4B, 4F, 5F, 6A-6S, and 7A-7G show the experimental data for endogenous proteins. These new data should strengthen our conclusions and make our conclusions more convincing. Importantly, the quality of the figures has also been improved. Figures 1D, 2A, 2B, 2D, 2E, 2F, 3A, 3D, 3G, 3H, 4A, 4B, 4C, 4D, 4E, 4F, 5B, 5C, 5E, 5F and the entire Fig. 6 and Fig. 7 have been newly added or modified during this revision. We hope these careful modifications to the manuscript adequately address the reviewer's concerns.

Comment 4: For the key experiment in fig. 7 (the only one that addresses some aspects of function in a rather contrived system) absolute values, rather than fold increases should be given. The data in panel A show disconcerting variation in the levels of Smurf1 and FBXL15 when assessed relative to each other. These experiments should include other members of the FBXL family as well.

R: In this revised manuscript, as we described above, we provide strong evidence to show the
The physiological role of FBXL15 in mammalian cells (Fig. 4), zebrafish embryos (Fig. 6) and adult rat bones (Fig. 7), thereby indicating the in vivo function of FBXL15 and the significance of FBXL15 in Smurf1 stability control.

For the exhibition of the reporter assay data, we found that most of the literatures used the fold increases. The reporter activity represents the firefly luciferase activity normalized by the renilla luciferase activity. We hope this explanation can address the reviewer's concern.

For the previous Fig. 7A (now the revised Fig. 4A), we have provided new data of higher quality. Regarding the possible effect of other members of the FBXL family on BRE activity, we have provided new data to show the specificity of FBXL15 (please see Supplementary Figure S3).

Comment 5: Papers that deal with the Ub-proteasome system increasingly show a tendency to extremely formulaic introductions: almost all of them read the same, and those in the field could skip page 1 without missing relevant information. For the TGF experts, the same is true for p. 2, and this part can be omitted almost entirely since the subject of TGF signaling itself hardly makes an appearance in the experimental section (exception fig 7). The text should be checked for proper idiomatic usage (agreement of subject and verb; dropped articles, superfluous articles, etc. etc.) of the English language by a native speaker.

R: We appreciate the reviewer’s criticisms. According to your useful suggestion, we have re-written the manuscript, and the organization, the descriptions and the logic of the manuscript have been significantly improved. We hope the introduction is more relevant to our own story, rather than a general introduction to Ub-proteasome or TGF signaling. The manuscript has also been edited by a professional language editing company, American Journal Experts (http://www.journalexperts.com). We hope these major revisions to the writing have adequately addressed the reviewer's concerns.

We deeply hope that these modifications will satisfy you and all of the referees and that the current manuscript will be considered for publication in your journal. Thank you very much in advance for your attention to our work. We look forward to your response.
REFeree reports:

Referee #1:

In this paper Cui et al. demonstrate that SCF(FBXL15) interacts with and ubiquitinates the ubiquitin ligase Smurf1. They characterize the interaction, demonstrate co-localization, show that by RNA interference that endogenous FBXL15 is involved in Smurf1 destabilization. Moreover, they provide evidence in a Zebra fish model that FBXL15 is involved in embryonic dorsalization; moreover it is shown in rats that FBXL15 siRNA treatment in bones causes loss of bone mass and a decrease in bone mineral density. As compared with the originally submitted paper this work is very much improved and the previous concerns have been addressed. I just have a few comments and questions:

1) Fig. 1C. The authors claim in the text (page 7) to this figure that pre-immune IgG fail to immunoprecipitate any FBXL15, whereas there is clearly some FBXL15 coming down. The authors should carry out this co-ipt under more stringent conditions.

2) Fig. 2E, turnover of Smurf1. Why are there so big differences between the 2 siRNAs for the turnover of Smurf1, despite the fact that the suppression of FBXL15 very comparable. This makes one wonder if these are really specific effects. May be the authors should reconstitute the silenced cells with a siRNA resistant FBXL15 construct.

3) Fig. 4B, page 13. The authors are pretending that depletion of FBXL15 leads to decreased Smad 1/5 levels. This is not obvious on this blot. Quantification may be required. Moreover, the blot for FBXL15 has to be shown also in this experiment.

4) No statistics (significance tests) are provided for any quantitative experiments

Referee #2:

Smurf proteins function as ubiquitin E3 ligases and play important roles in the regulation of BMP signal in bone remodeling as well as other signals during development. In this manuscript, Cui and colleagues attempted to investigate the mechanism of how the stability and activity of Smurf1 was regulated. Through a yeast-two-hybrid screen, they isolated a Smurf-interacting protein, FBXL15, an F-box protein of the FBXL. The authors provided evidence to support that FBXL15 acts as a positive regulator in BMP signaling by targeting smurf1 for its ubiquitination and degradation. The authors also examined biological functions of FBXL15 in zebrafish embryo and in rat bone tissue and thus generalized their conclusion.

In general, the topic of this manuscript is of potential interest. However, the data provided in the paper is too preliminary to support the main conclusion as the authors claimed.

I have listed my concerns in below:

1. In sections of abstract, introduction and discussion, the author claimed that "we show that FBXL15 forms a functionally active SCF complex and specifically targets Smurf1 for ubiquitination and proteasome-dependent degradation...". However, in Results, the authors did not provide any direct evidence to support that SCF complex is involved in Smurf1 ubiquitination and degradation as well as in regulating BMP signaling. Therefore, to reach their conclusion, the authors should provide evidence that the core components of SCF (e.g. Cullin1 and Roc1) are required for the ubiquitination and degradation of Smurf1 and modulating BMP activity in cell cultures.

2. In zebrafish embryo and in rat bone tissue experiments, the author should demonstrate that FBXL15 play roles in the determination of D/V patterning in zebrafish and rat bone homeostasis in a manner that depends on SCF complex activity.

3. Given that the E3 activity of Smurf proteins also contributes to the auto-ubiquitination themselves, the data in the current stage is not sufficient to exclude the possibility that FBXL15 functions as an adaptor to change the structure of Smurf1 for its auto-ubiquitination.

4. In the zebrafish experiments, the authors should use a second morpholino to demonstrate the specificity of FNXL15 knockdown by morpholinos. In addition, the quantitative PCR for
quantifying the relevant marker gene expression should be performed to evaluate the specific role of FNXL15 in D/V patterning formation when morpholinos are used.

5. In ubiquitination assay in cell cultures, FBXL15 knockdown should be included to see whether knockdown of FBXL15 indeed reduces the Smurf1 ubiquitination. Again, the author should exclude a possibility that FBXL15 regulates Smurf1 ubiquitination in a Smurf2-dependent manner.

6. In Figure 3C, the authors should use the GST protein as a negative control, since the endogenous GST protein exists abundantly in many tissues.

7. In Figure 6T, the authors found that co-injection of FBXL15 mRNA apparently down-regulate Smurf1 in zebrafish embryos. The question should be addressed is whether knockdown of FBXL15 could enhance smurf1-gfp fluorescence?

8. The SCF complex has been shown to play a role in controlling cell cycle progress, does the stability of Smurf1 have any cell-cycle dependence?

9. Why did FBXL15 stability in fig2E and fig3F exhibit different patterns, since the two experiments were performed in the same conditions.

Referee #3:

Cui et al, MS 2011-77128

The MS has improved substantially since the previous submission. Most importantly, it provides evidence for biological importance of FBXL15 using 2 in vivo models (zebrafish development and bone homeostasis in adult rats), and provides strong evidence that he Smurf proteins are the major targets of regulation by FBXL15.

There are several issues that still need to be addressed, however:

1. The ubiquitination figures are still of rather poor quality and some of the claims made that refer to them are not consistent with what is actually seen. Examples:
   (i) On page 10 (and Fig 3A): Smurf1(CA) is less ubiquitinated than WT-Smurf1, in spite of claim to the contrary. This same evidence of Smurf1 self-ubiquitination is also seen in Fig 3C. The issue of Smurf12 self-ubiquitination vs its ubiquitination by FBXL15 needs to be addressed.
   (ii) Fig 3C: The deltaF lane shows apparent loss of ubiquitination, but there is hardly any deltaF protein expressed there, hence it is hard to conclude that ubiquitination is lost.
   (iii) Fig 5C: This ubiquitination blot is of particular bad quality and also WWP2 does show increased ubiquitination in the presence of FBXL15, compatible with the loss of the protein shown in Fig 5B (and in contrast to the authors claims in several places in the text). How can that be explained given that WWP2 does not have the conserved Lys at the WH loop? Also, the authors’ previous submission showed strong ubiquitination of Nedd4-1 by FBXL15, but here it is weak. These discrepancies should be addressed.

2. Fig 6 (zebrafish experiments):
   (iv) The in situ data (Fig 6A-D) need to be repeated to include a “sense” control, and also to improve the quality of the in situ itself, since as it stands it is hard to see the pattern of expression claimed.
   (v) A second morpholino is needed for these experiments, to ensure no off target effects (this cannot be addressed by p53 MO).
   (vi) The quantification in Fig 6S is unclear, appear qualitative and no statistics are provided.
   (vii) There is no quantification of the phenotypic defects shown in Fig 6, panels F-I (ie the frequency of the defect and the number of embryos analyzed)

3. Fig 4B: the claim that Smad1/5 levels are decreased (page 13) is not supported by the data shown.

4. The Discussion is too long and can be shortened significantly without loss of information.
Responses to the Editor and the Referee #2:
Question from referee #2: The authors have addressed most of my comments. It appears that the current version of the manuscript was greatly improved. I only have one minor concern. Since the authors don't have direct evidence to exclude the possibility of ubiquitination of Smurf1 by Smurf1 itself or by Smurf2, they should discuss the potential issue that FBXL15 probably coordinates with Smurfs to control the stability of Smurf1 in Discussion.

Reply: Many thanks for your support. We agree with your suggestion and modified the related descriptions on Results section of Figures 3E & S3, and Discussion section.

Result section:

"Notably, FBXL15 seems not function as an adaptor to change the structure of Smurf1 for its auto-ubiquitination since incubation with GST alone or GST-FBXL15 protein had no significant effects on Smurf1 auto-ubiquitination under the in vitro ubiquitination conditions (Figure 3E, lanes 3 and 4). As a positive control, incubation with the Smurf1 cofactor CKIP-1 (Lu et al, 2008) resulted in a dramatic increase of Smurf1 auto-ubiquitination in vitro (lane 5). In addition, Smurf2 depletion in HEK293T cells had no significant effect on the FBXL15 activity of regulating Smurf1 ubiquitination (Supplementary Figure S3), implicating that FBXL15 might regulate Smurf1 ubiquitination in a Smurf2-independent manner" [Note: the previous description of this sentence is: excluding a possibility that FBXL15 regulates Smurf1 ubiquitination in a Smurf2-dependent manner].

I n the Discussion, the second paragraph, we added the below underlined sentences.

"To our knowledge, this is the first case to show a typical HECT E3 (Smurf1) acts as a substrate to be degraded in trans by a RING E3, especially by a multi-subunit SCF complex. Under the in vitro ubiquitination assay system, FBXL15 alone seems not to change the auto-ubiquitination ability of Smurf1 itself whereas the previous identified Smurf1 co-factor, CKIP-1 (Lu et al, 2008), can do it (Figure 3E). Also, depletion of Smurf2 in human embryonic kidney HEK293T cells also had no significant effects of the FBXL15 on Smurf1 ubiquitination (Supplementary Figure S3). However, we cannot rule out the possibility that the SCF<sup>FBXL15</sup> ligase complex coordinates with Smurf1 to control the ubiquitination and stability of Smurf1 itself, since the purified Cullin1/Roc1/Skp1 complex in the ubiquitination assays of this study (Figure 3D) might also co-purify the endogenous FBXL15 and Smurf1 proteins. In addition, we cannot rule out the possibility that SCF<sup>FBXL15</sup> complex might coordinate with Smurf2 to control Smurf1 stability in certain cell types or pathophysiological conditions. In this regard, a recent study showed that Smurf2 interacts with Smurf1 to induce the degradation of Smurf1 to prevent migration of breast cancer cells (Fukunaga et al, 2008). Previous studies have shown that RING E3s can be targeted by certain HECT E3s." We hope these modifications can satisfy the reviewer.

Question from the Editor: From the editorial perspective, I noticed some splicing of gels (particularly obvious in Figure B) and ask you to clearly mark distinct origins in this compilation by a white space or black bar.

Reply: Thanks for your suggestion. We have modified the gel images as you requested, particularly the Figure 6B. We added the IP-IB labels in Figure 1G. In addition, we also made some minor revisions on the references, and supplementary materials.

We deeply hope that these modifications will satisfy you and the referees. Thank you very much in advance for your attention to our work. We look forward to your response.