**The EMBO Journal (2013) 32,** 996–1007
www.embojournal.org

SOURCE DATA | TRANSPARENT PROCESS

THE EMBO JOURNAL

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

**Xiaofei Zhang[1], Juan Zhang[1], Andreas Bauer[2], Long Zhang[1], Douglas W Selinger[3], Chris X Lu[4] and Peter ten Dijke[1,*]**

[1]Department of Molecular Cell Biology, Cancer Genomics Centre Netherlands and Centre of Biomedical Genetics, Leiden University Medical Center, Leiden, RC, The Netherlands, [2]Novartis Pharma AG, Basel, Switzerland, [3]Developmental and Molecular Pathways, Novartis Institutes for BioMedical Research, Cambridge, MA, USA and [4]China Novartis Institute for Biomedical Research, Shanghai, China

**SMAD6 is a crucial feedback inhibitory regulator of bone morphogenetic protein (BMP)/SMAD signalling. Although little is known regarding the post-transcriptional modification of inhibitory SMADs and the mechanism by which their function is regulated. In this study, using a whole proteomic interaction screen for SMAD6, we identified a large putative E2 ubiquitin-conjugating enzyme UBE2O (E2-230K) as a novel interacting protein of SMAD6. We showed that UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6 at lysine 174 and that the cysteine 885 residue of human UBE2O is necessary for SMAD6 monoubiquitination. Inactivation of the SMAD6 monoubiquitination site specially potentiates the inhibitory ability of SMAD6 against BMP7-induced SMAD1 phosphorylation and transcriptional responses. We also found that UBE2O potentiated BMP7 signalling in a SMAD6-dependent manner. Addressing the molecular mechanism by which UBE2O and monoubiquitinated SMAD6 potentiate BMP7 signalling, we demonstrated that monoubiquitinated SMAD6 impairs the binding affinity of non-modified SMAD6 to the BMP type I receptor. Moreover, UBE2O and SMAD6 cooperated in the regulation of BMP7-induced adipogenesis.**

*The EMBO Journal* (2013) **32,** 996–1007. doi:10.1038/emboj.2013.38; Published online 1 March 2013
*Subject Categories:* signal transduction; differentiation & death
*Keywords*: adipocyte differentiation; BMP; monoubiquitination; SMAD6; UBE2O

## Introduction

Bone morphogenetic proteins (BMPs) are secreted proteins that belong to the transforming growth factor (TGF)-β family. BMPs are identified according to their ability to induce bone and cartilage formation, and in recent years that the broader importance of BMP signalling has been demonstrated (Wozney *et al*, 1988; Chen *et al*, 2004; Sieber *et al*, 2009; Lowery and de Caestecker, 2010; Miyazono *et al*, 2010; Ruschke *et al*, 2012). BMPs have been implicated in embryogenesis and development through their controlling of cell proliferation, differentiation, and apoptosis (Nohe *et al*, 2004; Miyazono *et al*, 2010; Plouhinec *et al*, 2011). They induce the formation of specific heteromeric complexes of type I and type II serine/threonine kinase receptors, upon which the BMP type I receptors, also termed activin receptor-like kinases (ALKs), can become phosphorylated at specific serine and threonine residues by the type II receptor kinase. The BMP type I receptor determines signalling specificity, and the activated type I receptor delivers the intracellular signal by phosphorylating receptor-regulated SMADs (R-SMADs), including SMAD1/5/8. Activated R-SMADs then translocate to the nucleus after forming heteromeric complexes with common partner-SMAD (Co-SMAD, i.e. SMAD4), where they regulate transcription of target genes (Sieber *et al*, 2009; Wu and Hill, 2009). One of the BMP signalling target genes is inhibitory (I-)SMAD6, has been shown to negatively regulate the BMP signalling (Itoh and ten Dijke, 2007).

UBE2O (Klemperer *et al*, 1989) and BRUCE (Bartke *et al*, 2004), are two relatively large E2 ubiquitin-conjugation enzymes in comparison with other E2s (14–35 kDa). UBE2O was first purified from rabbit reticulocytes and termed E2-230K (Klemperer *et al*, 1989). The ubiquitin-conjugating (UBC) domain of UBE2O was predicted to have E2 activity on the basis of sequence alignment with other known E2s (Yokota *et al*, 2001). Tissue expression of UBE2O is ubiquitous but occurs preferentially in brain, skeletal muscle, and heart tissues (Yokota *et al*, 2001). UBE2O is also expressed in erythroid cells, and is upregulated during the reticulocyte stage of erythroid differentiation (Haldeman *et al*, 1995; Wefes *et al*, 1995). Recently, UBE2O was shown to negatively regulate TRAF6-mediated NF-κB activation independent of its E2 activity (Zhang *et al*, 2013), which raises the question as to whether UBE2O indeed possesses E2 ubiquitin conjugase activity. In the present study, using a whole proteomic protein–protein interaction screen, we identified UBE2O as a novel interaction protein for SMAD6. We also showed that UBE2O monoubiquitinates SMAD6 and identified the E2 active site of UBE2O. In addition, our results support the hypothesis that UBE2O functions as an E2-E3 hybrid in SMAD6 monoubiquitination (Berleth and Pickart, 1996). We also found that upon SMAD6 monoubiquitination by UBE2O, SMAD6 displays reduced binding affinity for the activated BMP type I receptor ALK2. Finally, monoubiquitination of SMAD6 by UBE2O fine-tuned BMP7-induced adipocyte differentiation.

*Corresponding author. Department of Molecular Cell Biology, Cancer Genomics Centre Netherlands and Centre of Biomedical Genetics, Leiden University Medical Center, Postbus 9600, Leiden, 2300 RC, The Netherlands. Tel.: +31 71 526 9269; Fax:+31 71 526 8270; E-mail: p.ten_dijke@lumc.nl

**Received: 13 September 2012; accepted: 1 February 2013; published online: 1 March 2013**

## Results

### *Identification of UBE2O as an interacting protein of SMAD6*

SMAD6 and SMAD7 play key roles in inhibiting BMP- and/or TGF-β-mediated signalling (Itoh and ten Dijke, 2007; Park, 2005). To pinpoint novel regulators of BMPs/TGF-β signalling via SMAD6 or SMAD7, we performed a whole proteomic tandem affinity purification (TAP) assay to identify previously unknown interacting proteins for SMAD6 and SMAD7. TAP-tagged SMAD6 or SMAD7 was transfected into HEK293T cells to immunoprecipitate interacting proteins (Figure 1A, Supplementary Table S1, and unpublished observations), and several known interacting proteins for SMAD6 and/or SMAD7 were confirmed in our screen (blue ovals in Figure 1A and highlighted material in blue in Supplementary Table S1) (Murakami *et al*, 2003; Komuro *et al*, 2004; Inamitsu *et al*, 2006; Al-Salihi *et al*, 2012). Among the putative SMAD6- or SMAD7-binding proteins that have not been previously identified, the large E2 enzyme UBE2O caught our attention (yellow oval in Figure 1A and Supplementary Table S1). UBE2O was annotated as an E2 enzyme and contains a conserved UBC domain (Yokota *et al*, 2001). Co-transfection of Flag-SMAD6 or SMAD7 in HEK293T cells confirmed the interaction with UBE2O-Myc (Figure 1B and C). We next focused on UBE2O in SMAD6-mediated signalling as we found that UBE2O specifically monoubiquitinated SMAD6 (see below), a type of ubiquitination not previously reported for I-SMADs. Of note, endogenous SMAD6 or UBE2O interacted with ectopic UBE2O or SMAD6, respectively (Figure 1D and E). Moreover co-expression of UBE2O and SMAD6 deletion constructs followed by immunoprecipitation indicated that the N-terminal region of UBE2O and the C-terminal region of SMAD6 were necessary for the interaction (Figure 1F and G). Given these findings, and using this whole proteomic screening approach, we identified UBE2O as a novel interaction partner of SMAD6.

### *UBE2O monoubiquitinates SMAD6 in vivo*

As predicted, UBE2O is an E2 enzyme (Berleth and Pickart, 1996; Yokota *et al*, 2001), and a ubiquitination assay for all SMADs showed that ubiquitination of only SMAD6 and SMAD7 was altered when UBE2O was present (Figure 2A). Because previous reports have described the polyubiquitination of SMAD7 (Koinuma *et al*, 2003; Zhang *et al*, 2012b), we investigated the novel monoubiquitination of SMAD6 by UBE2O. An HA antibody or FK2 antibody, which specifically recognises ubiquitinated proteins, revealed a very specific monoubiquitination pattern of SMAD6 when SMAD6 was co-transfected with UBE2O and HA-ubiquitin (Figure 2B). Furthermore, an *in vivo* nickel pull-down ubiquitination assay or *in vitro* ubiquitination assay revealed results consistent with the immunoprecipitation assay (Figure 2C and Figure 3B). Of importance, the depletion of UBE2O using two independent shRNAs reduced the level of monoubiquitinated SMAD6 (Figure 2D). Thus, we identified UBE2O as a critical determinant that mediates the monoubiquitination of SMAD6.

### *UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6*

Sequence alignment of UBE2O revealed the conservation of the UBC domain from *Arabidopsis thaliana* to *Homo sapiens* (Supplementary Figure S1A). To ensure that cysteine 885 within the UBC domain of human UBE2O represents the E2 active site, cysteine 885 (Figure 3A, top panel) was mutated to a serine and tested for its potential to mediate SMAD6 ubiquitination. As predicted, the mutant UBE2O was unable to monoubiquitinate SMAD6 (Figure 3A). Intriguingly, we found that for the deletion of UBE2O with E2 activity (D2), only the addition of the remainder of UBE2O (D1) or C885S *in vitro* resulted in the monoubiquitination of SMAD6 (Figure 3B). Moreover, an immunoprecipitation assay revealed the self-interaction of the N-terminal and C-terminal portions of UBE2O protein (Supplementary Figure S1B). These results suggest that UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6.

### *UBE2O monoubiquitinates lysine 174 of SMAD6*

To map the amino acid in SMAD6 that UBE2O modulates, we first used a SMAD6 mutant deficient in lysine residues to perform the ubiquitination assay of SMAD6 (Figure 4A). The monoubiquitination of SMAD6 by UBE2O was completely abolished when all lysines in SMAD6 were mutated to arginines (Figure 4B); we observed a specific modification band, which was smaller than the monoubiquitinated SMAD6 band, when all lysine residues were mutated. However, this modification was dependent on forced ubiquitin but not UBE2O expression. Next, we mutated lysine residues to arginine in different regions of SMAD6 and narrowed the monoubiquitination site to the N-terminal region of the protein (Figure 4A and C). The ubiquitination assay for single or double lysine(s) mutated in the N-terminal portion of SMAD6 showed that the residue 174 lysine-to-arginine mutant displayed monoubiquitination levels that were markedly reduced as compared with wild-type SMAD6 (Figure 4D). Thus, lysine 174 of SMAD6 is the site at which monoubiquitination occurs via UBE2O.

### *Monoubiquitination of SMAD6 enhances BMP7-induced signalling*

To investigate the function of monoubiquitinated SMAD6, we screened all known reported signalling pathways that could be affected by SMAD6 (Supplementary Figure S2A–S2G). Of these signalling pathways, BMP7-induced BMP/SMAD response element (BRE) reporter activity was decreased when lysine 174 was mutated to arginine in comparison, with wild type or the nearby lysine (K182)-mutated SMAD6; this inhibitory effect was dose-dependent (Figure 5A). Moreover, BMP7-induced SMAD1 phosphorylation was inhibited more severely upon mutation of SMAD6 lysine 174 (Figure 5B). Consistent with the previous findings (Neumann *et al*, 2007; Tseng *et al*, 2008), the pre-treatment of C3H10T1/2 mesenchymal stem cells with BMP7 enabled their commitment to pre-adipocytes and differentiation to adipocytes, and BMP type I receptor kinase activity was required for this response (Supplementary Figure S3A). We also found that the K174R SMAD6 mutant more potently inhibited BMP7-induced adipocyte differentiation as compared with wild-type SMAD6 (Figure 5C). Furthermore, these results are consistent with the effects observed for the K174R SMAD6 mutant versus wild-type SMAD6 on BMP7-induced adipocyte genes expression levels (Figure 5D). Interestingly, we found that UBE2O protein levels increased upon BMP7-induced commitment to the pre-adipocyte stage (Figure 5E and F); this increase in UBE2O expression levels

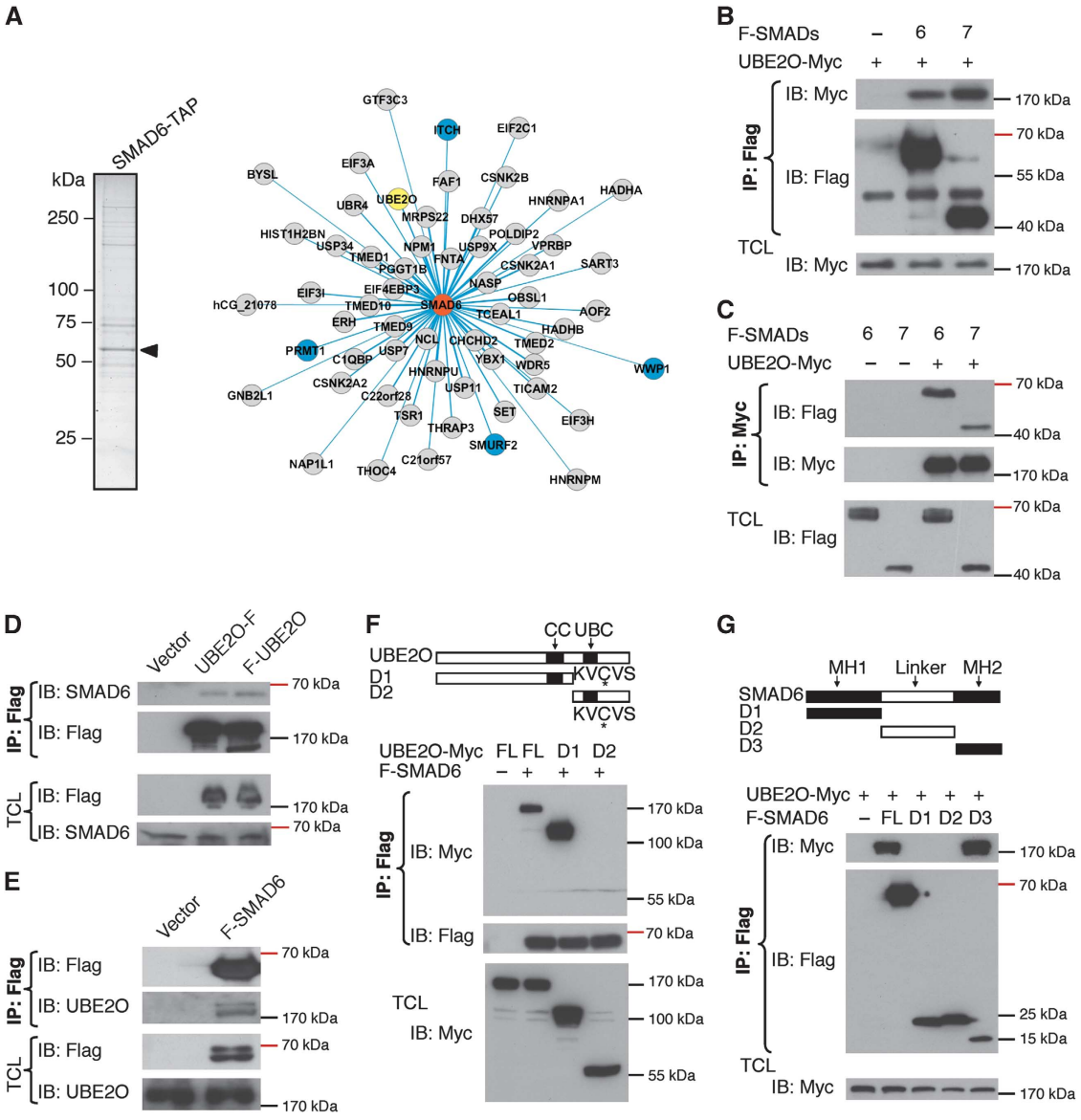



**Figure 1** Identification of UBE2O as a SMAD6-interacting partner. (**A**) Proteomic screening of interacting protein for SMAD6. HEK293T cells transfected with TAP-SMAD6 were lysed for immunoprecipitation. Purified protein complexes were separated by SDS–PAGE and stained by colloidal Coomassie Blue. Triangle indicates accumulation of SMAD6. Blue or grey ovals in right panel indicate the known or unknown interacting protein for SMAD6, UBE2O is highlighted in yellow. The graph was created using Cytoscape's Edge-Weighted Spring Embedded layout using the Fisher exact *P*-values as weights, such that more significant interactors will tend to be closer to SMAD6. Thicker edges represent higher significance (Smoot *et al*, 2011). (**B**, **C**) Interaction between UBE2O and SMAD6 or SMAD7. HEK293T cells were transfected with Flag-SMAD6, Flag-SMAD7 alone or with UBE2O-Myc as indicated for 48 h. Cells were harvested for immunoprecipitation with anti-Flag (**B**) or anti-Myc (**C**) resin. Myc (**B**) or Flag (**C**) antibody was used to detect the interaction. (**D**, **E**) UBE2O is a SMAD6-interacting protein. HEK293T cells were transfected with C-terminal- or N-terminal-Flag-tagged UBE2O (**D**) or Flag-tagged SMAD6 (**E**) for 48 h. Cells were harvested for immunoprecipitation with anti-Flag resin. SMAD6 (**D**) or UBE2O (**E**) antibody was used to detect the interaction. (**F**) The N-terminal of UBE2O interacts with SMAD6. (Top panel) Schematic illustration of the deletion mutants of UBE2O. (Bottom panel) HEK293T cells were transfected with Flag-SMAD6, UBE2O-Myc, or its deletions as indicated for 48 h. Cells were harvested for immunoprecipitation with anti-Flag resin. Myc antibody was used to detect the interaction. (**G**) The MH2 domain of SMAD6 interacts with UBE2O. (Top panel) Schematic illustration of the deletion mutants of SMAD6. (Bottom panel) HEK293T cells were transfected with Flag-SMAD6, SMAD6 deletions with UBE2O-Myc as indicated for 48 h. Cells were harvested for immunoprecipitation with anti-Flag resin. Myc antibody was used to detect the interaction. IP, immunoprecipitation; IB, immunoblot; TCL, total cell lysate. Source data for this figure is available on the online supplementary information page.

may function to limit the inhibitory effect of SMAD6. The latter was also induced but with delayed kinetics as compared with UBE2O. Upon the removal of BMP7 and the addition of adipocyte-favoring culture conditions (see Materials and Methods), we found that the UBE2O levels were markedly reduced, thereby illustrating the dynamic regulation of UBE2O expression (Figure 5E and F). Taken together, our findings suggest that UBE2O monoubiquitinates SMAD6 to attenuate its inhibitory effects and thus augments BMP7-induced signalling in adipocyte differentiation.

### *Monoubiquitinated SMAD6 decreases the binding of SMAD6 to the activated BMP type I receptor*

BMP7 activates signalling primarily via the BMP type I receptor ALK2 (Macias-Silva *et al*, 1998), and co-transfection with SMAD6 resulted in a strong interaction

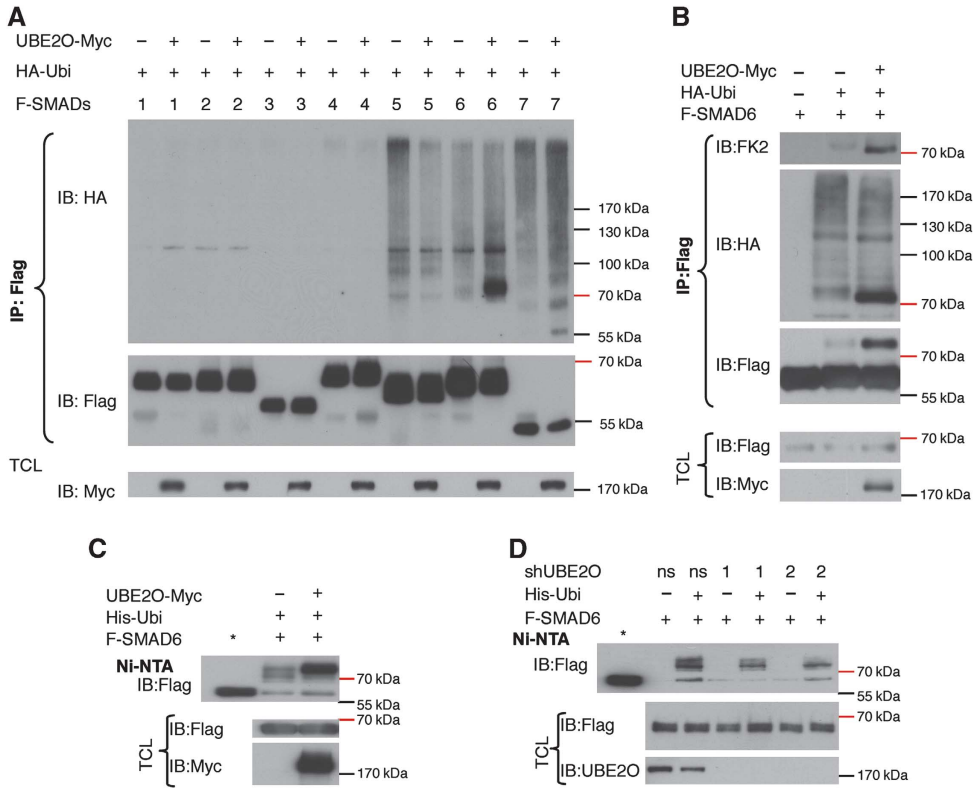



**Figure 2** UBE2O monoubiquitinates SMAD6. (**A**) UBE2O specifically ubiquitinates SMAD6 and SMAD7. HEK293T cells were transfected with indicated Flag-SMADs and HA-ubiquitin with or without UBE2O-Myc for 48 h. *In vivo* ubiquitination of SMADs proteins was performed by immunoprecipitation. Cells were sonicated with 1% SDS, then diluted to 0.1% SDS for immunoprecipitation with anti-Flag resin. HA antibody was used to detect ubiquitinated SMADs. (**B**, **C**) UBE2O monoubiquitinates SMAD6 *in vivo*. HEK293T cells were transfected with Flag-SMAD6, HA-ubiquitin (**B**) or His-ubiquitin (**C**) and UBE2O-Myc for 48 h. (**B**) *In vivo* ubiquitination of SMAD6 by immunoprecipitation was performed as described in panel **A**. Monoubiquitinated SMAD6 was detected by HA or FK2 (specifically recognise ubiquitinated protein) antibody. (**C**) Cells were lysed in 8 M urea buffer, and nickel affinity resin was used to perform the pull down (Ni-NTA); monoubiquitinated SMAD6 was detected by Flag antibody. Asterisk indicates input of SMAD6. (**D**) Depletion of UBE2O decreases monoubiquitination of SMAD6 *in vivo*. HEK293T cells stably expressing UBE2O shRNA (sh*UBE2O*-1, -2) were transfected with Flag-SMAD6 and His-ubiquitin for 48 h. Nickel pull down was performed as described in Figure 2C. Monoubiquitinated SMAD6 (ubi-SMAD6) was detected by Flag antibody. Asterisk indicates input of SMAD6. Source data for this figure is available on the online supplementary information page.

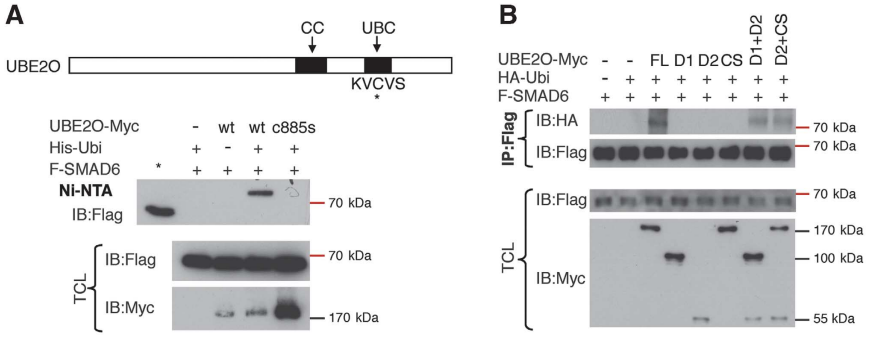



**Figure 3** UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6. (**A**) Cysteine 885 is the E2 active site of UBE2O for monoubiquitinating SMAD6. The conserved and putative E2 active site cysteine 885, located in the UBC domain of UBE2O, was mutated to serine. HEK293T cells were transfected with Flag-SMAD6, His-ubiquitin, and UBE2O-Myc or C885S-Myc for 48 h. Cells were lysed in 8 M urea buffer, and nickel affinity resin was used to perform the pull down. Flag antibody was used to detect monoubiquitinated SMAD6. Asterisk indicates input SMAD6. (**B**) UBE2O functions as an E2-E3 hybrid in SMAD6 monoubiquitination. Purified Flag-SMAD6, UBE2O-Myc or UBE2O deletions (described in Figure 1F) with Myc tag were incubated with E1 enzyme and HA-ubiquitin as described in Materials and Methods. HA antibody was used to detect monoubiquitinated SMAD6 after immunoprecipitation with anti-Flag resin. Source data for this figure is available on the online supplementary information page.

with constitutively active ALK2 (caALK2, Supplementary Figure S4A; (Goto *et al*, 2007)). We also found that monoubiquitination-defective SMAD6 was more potent at inhibiting BMP7-induced SMAD1 phosphorylation (Figure 5B). In addition, analysis of the subcellular distribution of (monoubiquitinated) SMAD6 protein via cell fractionation demonstrated that monoubiquitinated SMAD6 (like UBE2O) was predominantly localised in the cytoplasm, whereas non-modified SMAD6 protein was present in all subcellular fractions (Figure 6A and Supplementary Figure S4B).

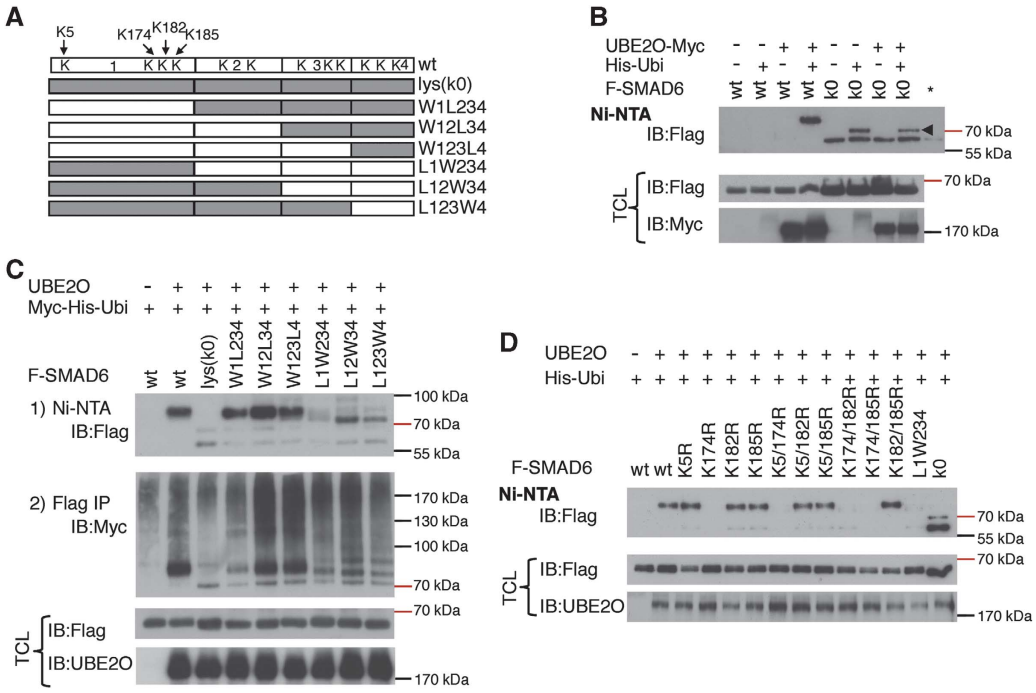



**Figure 4** Lysine 174 of SMAD6 is monoubiquitinated by UBE2O. (**A**) Schematic of the chimeric SMAD6. Wild-type (wt) and lysine-deficient (k0) SMAD6 were used to make chimeric SMAD6. SMAD6 was divided into four regions and chimeric SMAD6 was named according to presence of lysine. Example: W1L234 was made by fusing region 1 of wt SMAD6 and domain 2, 3 and 4 of k0 SMAD6. (**B**) Lysine is responsible for the monoubiquitination of SMAD6 by UBE2O. HEK293T cells were transfected with wild-type Flag-SMAD6 (wt) or lysine-deficient SMAD6 (k0), His-ubiquitin, and UBE2O-Myc for 48 h. Cells were lysed in 8 M urea buffer, and nickel affinity resin was used to perform the pull down. Flag antibody was used to detect monoubiquitinated SMAD6. Triangle shows the unspecific band when lysine-deficient SMAD6 was co-transfected with ubiquitin, which could be the N-terminal ubiquitination when all lysine were mutated in SMAD6. (**C**) N-terminal of SMAD6 is monoubiquitinated by UBE2O. HEK293T cells were transfected with the indicated Flag-SMAD6 (wild-type or chimeric SMAD6), Myc-His-ubiquitin, and UBE2O. Nickel pull down (described in Figure 4B) or immunoprecipitation with anti-Flag (described in Figure 2A) resin was used to identify the monoubiquitination region of SMAD6. (**D**) UBE2O monoubiquitinates lysine 174 of SMAD6. HEK293T cells were transfected with wild-type Flag-SMAD6 (wt), lysine-deficient SMAD6 (k0), or the indicated single- or double-mutated SMAD6, His-ubiquitin, and UBE2O for 48 h. Nickel pull down was performed as in Figure 4B. Source data for this figure is available on the online supplementary information page.

Moreover, in agreement with this latter finding, an artificial SMAD6-ubiquitin fusion protein was absent from the nucleus and present in the cytoplasm (Supplementary Figure S4B). These results suggest that monoubiquitinated SMAD6 functions in the cytoplasm and upstream of SMAD1 phosphorylation. To address the mechanism by which monoubiquitinated SMAD6 enhances the BMP7 responses, we sought to purify monoubiquitinated SMAD6 from HEK293T cells. However, we failed to isolate the pure monoubiquitinated SMAD6 because of its ability to interact with itself (Kawabata *et al*, 1998; Supplementary Table S1). An *in vitro* interaction assay using wild-type SMAD6 or purified monoubiquitinated SMAD6 with constitutively active ALK2 (caALK2) showed that the interaction was reduced upon SMAD6 monoubiquitination (Figure 6B and C). Thus, monoubiquitination of SMAD6 antagonises its inhibitory effect by mitigating the ability of SMAD6 to interact with the activated type I receptor.

### *UBE2O functions as a positive regulator in BMP7 signalling*

To examine whether UBE2O affects BMP7-induced responses, we examined the effect of UBE2O misexpression on BMP7-induced transcriptional responses. Knockdown of UBE2O using two independent shRNAs markedly constrained BMP7-induced signal activation and SMAD1 phosphorylation (Figure 7A and B). Moreover, wild-type UBE2O, but not the catalytically inactive mutant (C885S), potentiated BMP7-induced SMAD transcriptional responses in a UBE2O dose-dependent manner (Figure 7C). To further investigate the function of UBE2O, we established a stable cell line expressing UBE2O or C885S-UBE2O in C3H10T1/2 cells. BMP7-induced SMAD1 phosphorylation and adipocyte differentiation were potentiated upon forced expression of UBE2O, whereas the expression of catalytically inactive UBE2O did not display this effect (Figure 7D–F). As BMP plays an important role in osteoblast differentiation, we also examined the effect of UBE2O overexpression in C2C12 myoblasts that can be differentiated into osteoblast-like cells by BMP6. Forced UBE2O expression in C2C12 cells enhanced BMP6-induced SMAD1 phosphorylation and alkaline phosphatase activity (ALP), an early marker of osteoblast differentiation (Figure 7G and H).

To confirm that the effect of UBE2O on BMP7 signalling occurs via alternation of SMAD6 function, we created a UBE2O-overexpressing stable cell line in *SMAD6*-knockout fibroblast cells (Supplementary Figure S5). In line with the antagonistic function of SMAD6 in BMP signalling (Estrada *et al*, 2011), BMP7-induced SMAD1 phosphorylation was potentiated in *SMAD6*-knockout cells (Figure 7I and J). Notably, UBE2O increased BMP7-induced SMAD1

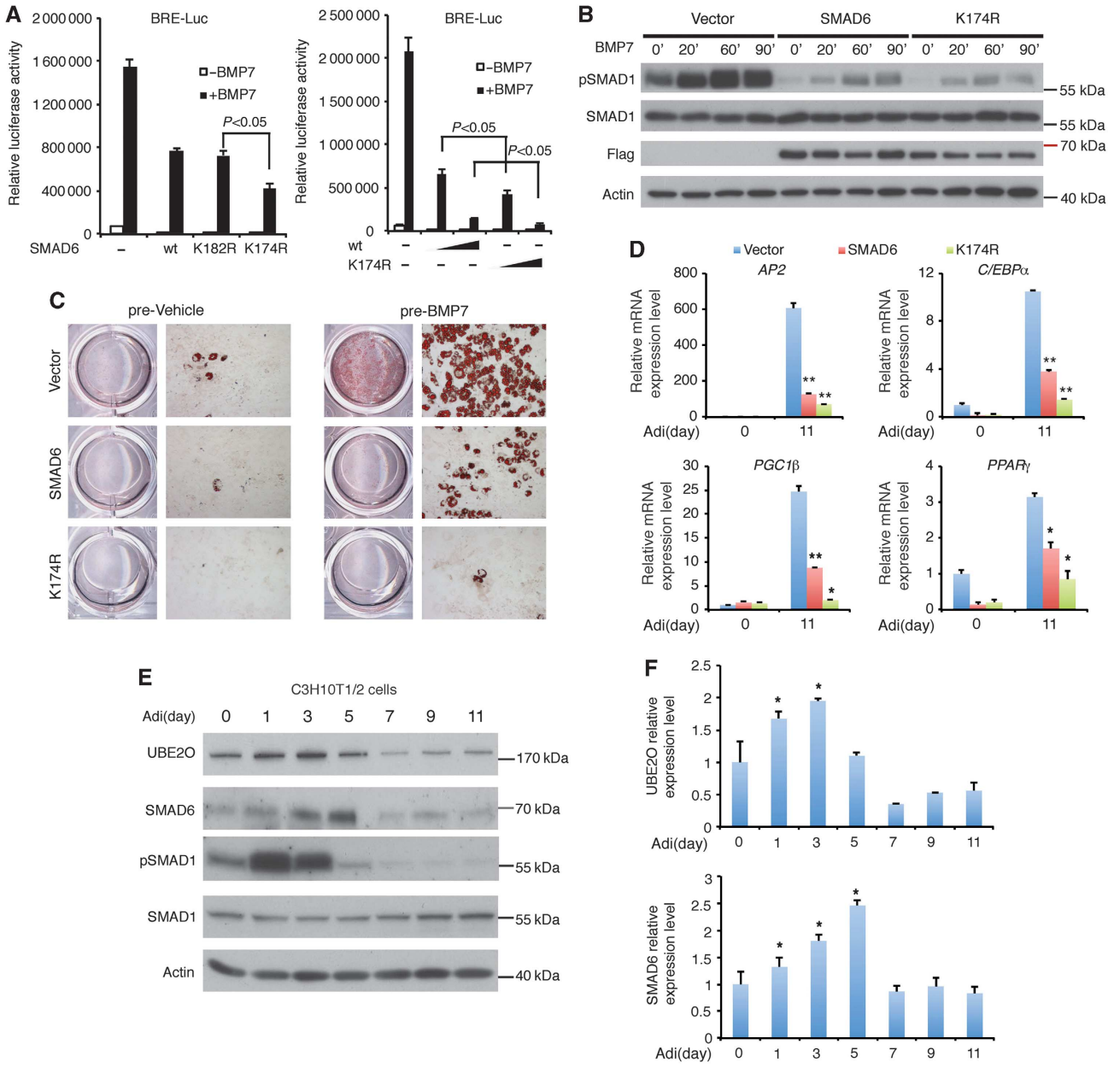



**Figure 5** Monoubiquitination of SMAD6 potentiates BMP7-induced signalling. (**A**) Lysine 174-mutated SMAD6 increases the inhibitory ability of SMAD6 on BMP7-induced signalling. Analysis of BMP7-induced signalling activation in HEPG2 cells with co-transfection of SMAD6 (wt), lysine 182 or 174 to arginine (K182R or K174R, left panel) or increase amounts of lysine 174 to arginine (K174R, right panel). Data from triplicates are presented as the mean ± s.d. of a representative experiment. (**B**) Lysine 174-mutated SMAD6 increases the inhibitory ability of SMAD6 on BMP7-induced SMAD1 phosphorylation. C3H10T1/2 cells stably expressing empty vector, SMAD6, or K174R were stimulated with BMP7 at the indicated time points, and western blots were used to analyse phosphorylated SMAD1 level (pSMAD1). (**C**) Lysine 174-mutated SMAD6 increases the inhibitory ability of SMAD6 on BMP7-induced adipocyte differentiation. C3H10T1/2 cells stably expressing empty vector, SMAD6, or K174R were pre-treated with BMP7 for 3 days and differentiated into adipocytes for another 8 days. Cells were fixed and stained with Oil Red O as described in Materials and Methods. Whole well and higher magnification are shown. (**D**) Lysine 174-mutated SMAD6 increases the inhibitory ability of SMAD6 on BMP7-induced adipocyte-related genes expression. C3H10T1/2 cells stably expressing empty vector, SMAD6, or K174R were pre-treated with BMP7 for 3 days and differentiated into adipocytes for another 8 days. Adi(day) represents adipocyte differentiation days. Adipocyte cell markers *AP2*, *CEBP/α*, *PGC1β*, and *PPARγ* were analysed by real-time PCR. *indicates $P<0.05$ and **indicates $P<0.01$ (wild-type SMAD6 was compared with empty vector and K174R mutated SMAD6 was compared with wild-type SMAD6). (**E**) SMAD6 and UBE2O are upregulated in BMP7-induced commitment of mesenchymal stem cells to proliferating pre-adipocytes. C3H10T1/2 cells were pre-treated with 100 ng/μl BMP7 in DMEM supplemented with 10% FBS for 3 days to reach confluence, cells were induced to adipocytes as described in Materials and Methods. Cells were harvested for western blot at the indicated time points. Adi(day) represents adipocyte differentiation days. (**F**) The relative protein level of UBE2O or SMAD6 (compared with protein level of UBE2O or SMAD6 without BMP7 treatment) was quantified. *indicates $P<0.05$. Source data for this figure is available on the online supplementary information page.

phosphorylation levels only in wild-type fibroblast cells and not in *SMAD6*-knockout fibroblast cells (Figure 7I and J). Thus, UBE2O potentiates BMP7-induced signalling, which depends at least in part on SMAD6 expression.

## Discussion

SMAD6 is a critical feedback regulator that limits BMP signalling, but, unlike other SMADs, little is known regarding its post-translational modification. Recently, several reports have

described the monoubiquitination of Co-/R-SMADs and the regulation of TGF-β signalling (Dupont *et al*, 2009; Inui *et al*, 2011). Unlike the polyubiquitination associated with protein degradation or signal complex formation, monoubiquitination alters R-SMAD and Co-SMAD complex formation or transcriptional complex assembly. The present observation contributes to this body of knowledge, as we demonstrated for the first time the monoubiquitination of an I-SMAD by UBE2O. Although UBE2O is an uncharacterised putative E2 enzyme, with little known about its function, we showed that UBE2O functions as an E2-E3 hybrid enzyme to monoubiquitinate SMAD6. We further found that monoubiquitination of SMAD6 attenuated its apparent binding affinity to the BMP type I receptor ALK2. Functionally, we revealed that the monoubiquitination of SMAD6 by UBE2O is involved in BMP7-induced adipocyte differentiation, and we established the coordination between SMAD6 and UBE2O in BMP7 signalling and BMP7-induced adipocyte differentiation (Figure 8).

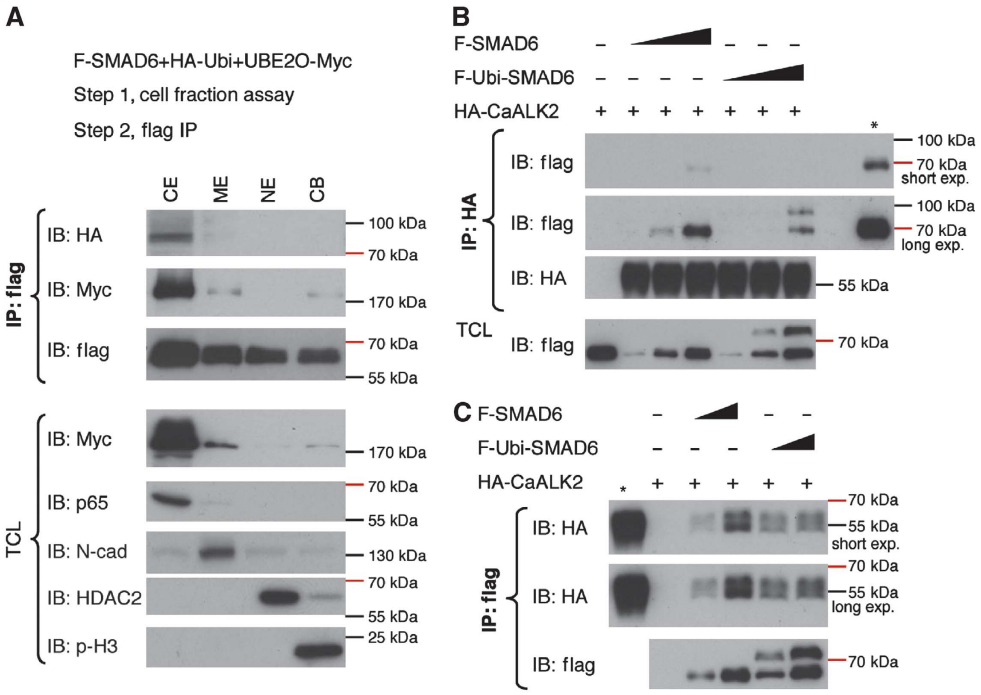


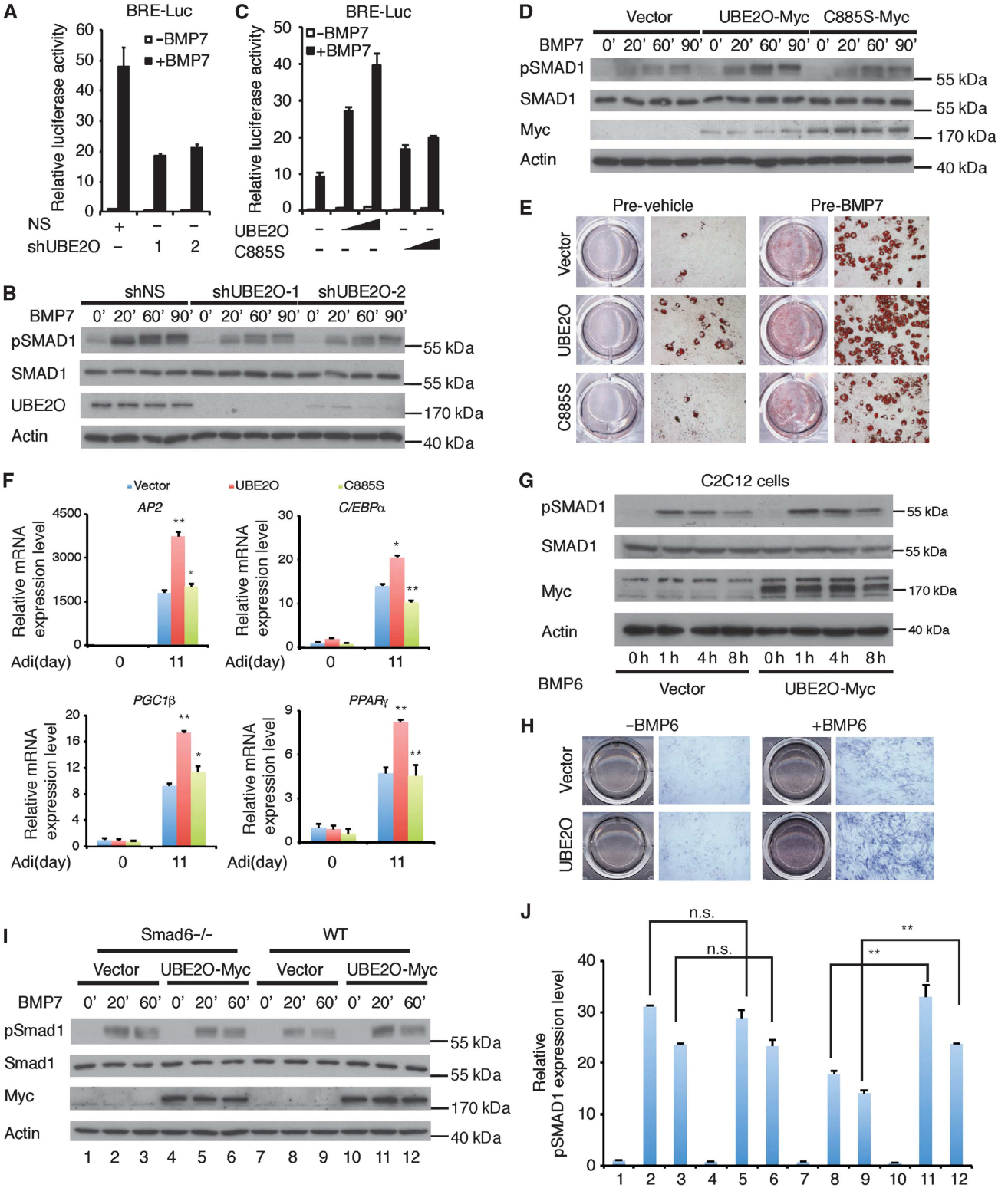

**Figure 6** Monoubiquitinated SMAD6 restrains the interaction of ALK2 and SMAD6. (**A**) Subcellular localisation of monoubiquitinated SMAD6. HEK293T cells were transfected with Flag-SMAD6, HA-ubiquitin, and UBE2O-Myc for 48 h. Cell fractionation assays were performed as described in Materials and Methods. Four endogenous proteins (p65 for cytoplasmic extract, N-cadherin for membrane extract, HDAC2 for soluble nuclear extract and phosphorylated Histone 3 for chromatin-bound extract) were used as markers for cell fractionation assay. *In vivo* ubiquitination of fractionated SMAD6 was performed by immunoprecipitation with Flag-resin. HA antibody was used to detect monoubiquitinated SMAD6. CE, cytoplasmic extract; ME, membrane extract; NE, soluble nuclear extract; CB, chromatin-bound extract. (**B, C**) Monoubiquitinated SMAD6 reduces the interaction between SMAD6 and caALK2. Purified Flag-SMAD6, Flag-ubiquitinated SMAD6 (F-Ubi-SMAD6), and HA-caALK2 from HEK293T cells were mixed together as indicated, and results of immunoprecipitation with HA- (**B**) or Flag- (**C**) resin were analysed with indicated antibodies. Asterisks indicate the input. Source data for this figure is available on the online supplementary information page.

**Figure 7** UBE2O potentiates BMP7-induced SMAD signalling. (**A, C**) UBE2O positively regulates BMP7-induced signalling activation. Analysis of BMP7-induced signalling activation in HEPG2 cells with knockdown of UBE2O (**A**), or with increased amounts of UBE2O or C885S-mutated UBE2O (**C**). Data from triplicates are presented as the mean ± s.d. of a representative experiment. NS represents non-specific shRNA. (**B, D**) UBE2O potentiates BMP7-induced SMAD1 phosphorylation. C3H10T1/2 cells stably expressing UBE2O shRNA (shUBE2O-1, -2) (**B**) or stably expressing UBE2O or C885S-mutated UBE2O (**D**) were stimulated with BMP7 at the indicated time points. Western blots were used to analyse phosphorylated SMAD1 level with indicated antibodies. (**E, F**) UBE2O potentiates BMP7-induced adipocyte differentiation. C3H10T1/2 cells stably expressing empty vector, UBE2O, or C885- mutated UBE2O were pre-treated with BMP7 for 3 days and differentiated into adipocytes for another 8 days. Cells were fixed and stained with Oil Red O as described in the Materials and Methods (**E**) or analysed by real-time PCR for adipocyte markers *AP2*, *CEBP/α*, *PGC1β*, and *PPARγ* (**F**). Adi(day) represents adipocyte differentiation days. *indicates $P<0.05$ and **indicates $P<0.01$ (wild-type UBE2O was compared with empty vector and C885S-mutated UBE2O was compared with wild-type UBE2O). (**G**) UBE2O potentiates BMP6-induced SMAD1 phosphorylation. C2C12 cells stably expressing empty vector or UBE2O-Myc were stimulated with 50 ng/ml BMP6 at the indicated time points. Western blots were used to analyse phosphorylated SMAD1 level with indicated antibodies. (**H**) UBE2O potentiates BMP6-induced osteoblast-like cell differentiation. C2C12 cells stably expressing empty vector or UBE2O-Myc were treated with 50 ng/ml BMP6 for 3 days, and then cells were harvested for histochemical staining to determine ALP activity. Whole well and higher magnification are shown. (**I**) The potentiated BMP7-induced signalling by UBE2O depends on SMAD6. Immortalised *SMAD6*-knockout MEF cells were infected with empty vector or UBE2O-Myc expression viruses. Cells were treated with BMP7 at the indicated time points. Phosphorylated SMAD1 level was analysed by western blot. (**J**) The relative BMP7-induced SMAD1 phosphorylation level in Figure 7G (normalised to SMAD1) was quantified. **indicates $P<0.01$, n.s. indicates no significant difference. Source data for this figure is available on the online supplementary information page.

### *UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6*

We identified UBE2O as a putative interacting protein of SMAD6 from a whole proteomic screen, and our results revealed that UBE2O interacts with and specifically mono-ubiquitinates SMAD6. Moreover, we found that inactivation of the conserved E2 active site completely inhibited the ability of UBE2O to promote SMAD6 monoubiquitination. Furthermore, we determined that lysine 174 of SMAD6 is responsible for the monoubiquitination of SMAD6 by UBE2O. However, we also observed monoubiquitination, as with modified SMAD6, and varying levels of polyubiquitinated SMAD6 in some chimeric SMAD6 molecules, which indicate that a compensation mechanism is involved in SMAD6 ubiquitination, such as ubiquitin shifts to lysine or other amino acids (Wang *et al*, 2007; Tokarev *et al*, 2011; Hagai



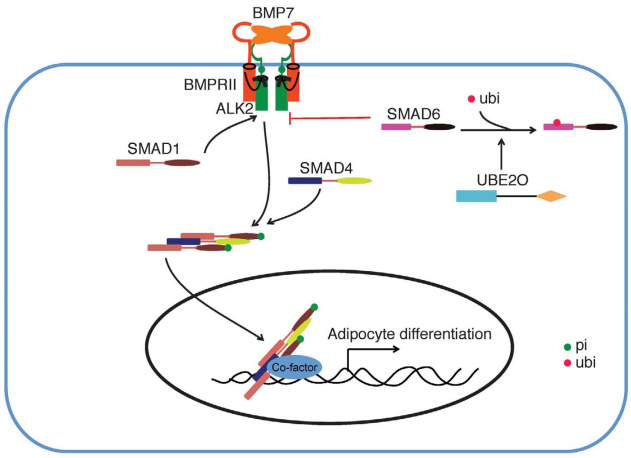



**Figure 8** Schematic shows how UBE2O potentiates BMP7 signalling. Without monoubiquitination, SMAD6 binds to activated type I receptor to inhibit BMP7-induced SMAD1 phosphorylation and subsequent regulation of transcriptional responses. Monoubiquitinated SMAD6 by UBE2O impairs the association of activated type I receptor with non-modified SMAD6, resulting in more prominent BMP7/SMAD signalling. pi represents phosphorylation and ubi represents ubiquitination.

*et al*, 2012). Further experiments, such as mass spectral-aided analysis of SMAD6 post-translational modifications will help to clarify this mechanism.

Among the E2s that have been identified, BRUCE has been suggested to function as an E2-E3 hybrid to monoubiqutinate SMAC (Bartke *et al*, 2004). UBE2O has also been suggested to function as an E2-E3 hybrid (Berleth and Pickart, 1996), whereby ubiquitin conjugated to an E2 active site cysteine is passed to another cysteine residue of UBE2O, which results in the transfer of ubiquitin from the latter cysteine residue to the substrate. Our SMAD6 *in vitro* ubiquitination assay results support this hypothesis. Moreover, we found that a C-terminal deletion mutant of UBE2O with E2 activity partially monoubiquitinated SMAD6 with the help of the remaining UBE2O, which suggests that the N-terminal portion of UBE2O may exert the E3 activity. Therefore, further investigations are needed to identify the cysteine residue that functions as an E3 active site.

### *The function of monoubiquitinated SMAD6 and UBE2O in BMP7 signalling*

SMAD6 plays an inhibitory role in several signal pathways, such as the TGF-β/BMP, WNT, and IL-1R pathways (Imamura *et al*, 1997; Choi *et al*, 2006; Xie *et al*, 2011). However, monoubiqutination-disabled SMAD6 exerts a significantly more potent antagonising effect on BMP7-induced signalling and SMAD1 activation, which suggests that monoubiquitinated SMAD6 specifically exerts weaker inhibitory activity on BMP7 signalling as compared with non-modified SMAD6. In line with these results, wild-type UBE2O, but not the catalytically inactive UBE2O, monoubiquitinated SMAD6 and potentiated BMP7 signalling.

In addition, we showed that the positive effect of UBE2O on BMP7 signalling was SMAD6-dependent, as UBE2O failed to potentiate BMP7-induced SMAD1 phosphorylation when SMAD6 was depleted. Although the UBE2O-mediated potentiation of BMP7-induced signalling was dependent on SMAD6, we could not exclude the possibility that UBE2O-induced polyubiquitination of SMAD7 also contributes to the enhanced BMP7-induced responses. Consistent with this notion, UBE2O interacted with ARKADIA and RNF12 (Markson *et al*, 2009 ; Supplementary Figure S6A), two E3 ubiquitin ligases that target SMAD7 for polyubiquitination and degradation (Koinuma *et al*, 2003; Zhang *et al*, 2012b). Interestingly, the SMAD7 ubiquitination assay demonstrated that UBE2O potentiates ARKADIA- or RNF12-induced polyubiquitination (Supplementary Figure S6B). Thus, UBE2O may potentiate BMP7 by acting as an E2-E3 hybrid by stimulating SMAD6 monoubiquitination and recruiting an E3 ligase to stimulate SMAD7 polyubiquitination. In conclusion, these findings indicate that UBE2O amplifies BMP7 signalling, at least in part, via SMAD6 monoubiquitination.

### *Monoubiqutinated SMAD6 has a lower affinity for binding to the BMP type I receptor*

Unlike polyubiquitination, which mainly triggers protein degradation or signal complex formation, substrate monoubiquitination frequently alters its localisation or interaction with other proteins (Tang and Zhang, 2011). Our results show that whereas wild-type SMAD6 was uniformly distributed throughout cells, monoubiquitinated SMAD6 was predominantly localised in the cytoplasm, as was also the case for UBE2O. Consistent with this result, the artificial SMAD6-ubiquitin fusion protein was also localised to the cytoplasm. Moreover, we found that SMAD6 displayed a lower binding affinity for the activated BMP7 type I receptor (ALK2), which provides a mechanism by which the monoubiquitination of SMAD6 functions less effectively as an antagonist of BMP7 signalling.

### *UBE2O and SMAD6 cooperate in BMP7-induced adipogenesis*

BMPs have many biological functions, including the induction of adipocyte differentiation (Neumann *et al*, 2007; Tseng *et al*, 2008), and our results showed that SMAD6 overexpression mitigated BMP7-induced adipocyte differentiation in the C3H10T1/2 mesenchymal stem cell line. Interestingly, monoubiquitination-deficient SMAD6 was more potent than wild-type SMAD6 in BMP7-induced adipocyte differentiation, which is consistent with the finding that monoubiquitinated SMAD6 exerts a weaker inhibitory effect on BMP7-induced signalling activation. In line with this observation, only wild-type UBE2O (but not the catalytically inactive UBE2O), which monoubiquitinates SMAD6 potentiated the adipocyte differentiation induced by BMP7. Moreover, both SMAD6 and UBE2O were dynamically upregulated with different kinetics during the BMP7-induced commitment of mesenchymal stem cells into proliferating pre-adipocytes. The earlier induction UBE2O by BMP7 may restrict the inhibitory function of SMAD6, thereby favoring pre-adipocyte commitment. This result is consistent with previous report that BMP activity was enhanced in proliferating pre-adipocytes and markedly weaker in differentiating adipocytes (Suenaga *et al*, 2010). Our results emphasise the physiological relevance of the interplay between UBE2O and SMAD6 for fine-tuning BMP signalling in pre-adipocyte commitment and adipocyte differentiation. In addition, we observed that UBE2O misexpression in C2C12 myoblast cells also affected

BMP6-induced SMAD1 phosphorylation and the differentiation into osteoblast-like cells. In conclusion, we identified UBE2O as a pivotal positive regulator of BMP signalling in mesenchymal stem cells.

## Materials and methods

### *Tandem affinity purification (TAP)*

A total of 19 'bait proteins' involved in the TGF-β/BMP pathway were TAP-tagged N- and C-terminally and used for affinity purification. Cells were harvested by mechanical detachment, washed with excess phosphate-buffered saline (PBS) on ice, and lysed in immunoprecipitation buffer essentially as described (Burckstummer *et al*, 2006). The isolated protein lysates were used as starting material for the TAP. Purified protein complexes were separated by one-dimensional SDS–PAGE and stained by colloidal Coomassie Blue. Entire gel lanes were systematically cut into slices, and proteins were in-gel digested with trypsin as described (Shevchenko *et al*, 1996). Protein identification by liquid chromatography-tandem mass spectrometry was done using an Eksigent 1D+ HPLC system coupled to an LTQ Orbitrap mass spectrometer (Thermo-Finnigan). Peptide mass and fragmentation data were used to query an in-house curated version of the International Protein Index (IPI) database using Mascot (Matrix Science).

Candidate lists were generated as follows. For each TAP-tagged bait-interactor pair, a $2 \times 2$ contingency table was constructed describing the number of purifications in which the interactor was observed with the bait versus other bait proteins. A two-tailed Fisher Exact test was then used to assess whether each interactor was observed significantly more (or less) frequently with a given bait compared to others tested. For this paper, we focus on SMAD6 interactors. Those proteins which were observed more frequently than expected are shown in Supplementary Table S1. Non-ribosomal proteins (ribosomal proteins are also found in many other 'bait proteins' interactome) with *P*-values $\leq 0.01$ are represented as a graph in Figure 1A. All IPI identifiers were mapped to Entrez Gene symbols prior to the Fisher Exact calculation.

### *Cell culture and expression plasmids*

HEK293T, C3H10T1/2, HEPG2, C2C12, immortalised wild-type or SMAD6-knockout MEFs (Estrada *et al*, 2011) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo) supplemented with 10% foetal bovine serum (FBS) (Hyclone) and 100 U/ml penicillin/streptomycin (Invitrogen). SV40 retroviruses were used to immortalise primary MEF cells as reported (Carracedo *et al*, 2010). For the chimeric SMAD6 plasmids, lysine-deficient SMAD6 (K0) and wild-type (WT) SMAD6 with engineered enzymatic sites without changes in amino acids were purchased from GenScript. To get chimeric SMAD6 expression plasmids, indicated region of WT (W) SMAD6 and lysine-deficient SMAD6 (L) were ligated to each other. Point mutations of SMAD6 or UBE2O were performed using KOD hot-start DNA polymerase from Merck. To produce viruses, UBE2O, UBE2O-C885S, SMAD6, and SMAD6-K174R were cloned into pLV-bc-CMV-puro lentivirus (pLV) vector with C-terminal Myc or N-terminal Flag tag. Detailed information about plasmids is described in Supplementary Data. Cloning primers used in this paper are available on request. All new constructs were confirmed by DNA sequencing.

### *Immunoblotting and immunoprecipitation assays*

Cells were lysed with 1 ml of TNE-lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40) plus a protease inhibitor cocktail (Roche) for 10 min on ice. After centrifugation at 13 200 r.p.m. 4 °C for 10 min, protein concentration was measured (DC protein assay, Bio-Rad), and equal amounts of lysates were used for immunoblotting analysis. Blots were quantified using ImageJ (NIH). For immunoprecipitation, cells were lysed with the same TNE-lysis buffer, and cell supernatants were incubated with Flag-Resin (A2220, Sigma) or HA-Resin (A2095, Sigma) at 4 °C for 2 h after centrifugation at 13 200 r.p.m. 4 °C for 10 min. After this, the precipitants were washed three times with TNE buffer, and bound proteins were boiled with loading buffer for 5 min and separated by SDS–PAGE. Western blotting was performed using the Bio-Rad mini-gel running system. Antibodies used in this study were c-Myc (a-14, sc-789, Santa Cruz Biotechnology), HA (Y-11, sc-805, Santa Cruz Biotechnology), Flag (F3165, Sigma), β-actin (A5441, Sigma), UBE2O (NBP1-03336, Novus Biologicals), SMAD6 (9519, Cell Signaling and sc-13048, Santa Cruz Biotechnology), SMAD1 (sc-7965, Santa Cruz Biotechnology, 6944, Cell Signaling and 060653, Upstate), pSMAD1 (9511, Cell Signaling), and FK2 (BML-PW8810, Enzo Life Sciences).

### *Transfections and stable cell line selection*

Cells were transfected with polyethylenimine (PEI, Sigma). Lentiviruses were used to obtain stable cell line as described before (Zhang *et al*, 2012a). Detailed information is described in Supplementary Data.

### *Protein purification and in vitro interaction*

To obtain purified monoubiquitinated SMAD6 (Flag-Ubi-SMAD6), Flag-SMAD6, 10× His-Ub, and UBE2O-Myc plasmids were transfected into HEK293T. Cell lysates in NP40 lysis buffer were immunoprecipitated with nickel beads for 3 h at 4 °C, followed by elution with NP40 lysis buffer with 1 M imidazole (Sigma). Pooled eluates were subsequently immunoprecipitated with anti-Flag resin, followed by elution with 100 μg/ml Flag peptide (Sigma) in *in vitro* binding buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 0.1% NP40, 5% glycerol). Flag peptides were discarded, and eluates were concentrated using Amicon Ultra 3K (Millipore). For purification of SMAD6, UBE2O, UBE2O deletion mutant proteins, or caALK2, each plasmid was transfected into HEK293T, and cell lysates were immunoprecipitated for 2 h with anti-Flag, anti-Myc, or anti-HA resin and eluted with Flag, Myc, or HA peptide (Sigma); proteins were concentrated using Amicon Ultra 3K (Millipore).

For *in vitro* protein–protein interactions, purified Flag-SMAD6 or Flag-Ubi-SMAD6 and HA-caALK2 proteins were diluted in binding buffer and immunoprecipitated with anti-Flag or anti-HA resin for 4 h at 4 °C. Immunoprecipitates were washed three times with the binding buffer and boiled with SDS containing running buffer for subsequent analysis by immunoblotting.

### *In vitro and in vivo ubiquitination*

*In vitro* ubiquitin assays were performed by incubating ubiquitin-activating enzyme E1 (Ube1 40 nM, E305 Boston Biochem), HA-ubiquitin (8 μM, U110 Boston Biochem), Flag-SMAD6, together with UBE2O-Myc, C885S-Myc or deletions at 37 °C for 3 h in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP). A total of 45 μl of the 50-μl reaction system was diluted with RIPA buffer and followed with anti-Flag resin immunoprecipitation. A total of 5 μl of the 50-μl reaction system was diluted and boiled with SDS loading buffer. For *in vivo* ubiquitination analysis via immunoprecipitation (Zhou *et al*, 2012), cells were washed twice in cold PBS with 10 mM *N*-Ethylmaleimide (NEM) and lysed in 1% SDS-RIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with protease inhibitors and 10 mM NEM. Lysates were sonicated, boiled for 5 min, diluted to 0.1% SDS by RIPA buffer, and centrifuged at 13 200 r.p.m. at 4 °C for 10 min. Supernatants were incubated with anti-Flag resin for 2 h at 4 °C. After three washes with RIPA buffer, beads were boiled with loading buffer for 5 min and separated with SDS–PAGE. For *in vivo* ubiquitination analysis by nickel pull down (Ni-NTA), a modified method was used (Tatham *et al*, 2009). Cells were washed two times in cold PBS with 10 mM NEM and lysed in 6 ml 8 M urea buffer (8 M urea, 0.1 M $Na_2HPO_4$, 0.1 M $NaH_2PO_4$, 10 mM Tris-HCl, 10 mM imidazole, 10 mM β-mercaptoethanol). Lysates were centrifuged at 4000 r.p.m. for 10 min at room temperature and incubated with nickel beads at room temperature for 2 h. The beads were washed three times with 8 M urea buffer with 20 mM imidazole and boiled with loading buffer for 5 min before analysis with SDS–PAGE.

### *Cell fractionation assay*

For locating monoubiquitinated SMAD6 in cell lysates, a subcellular protein fractionation kit from Pierce (78840) was used, following the accompanying protocol.

### *Quantitative real-time RT–PCR*

RNA extraction was performed using NucleoSpin RNA II (MACHEREY-NAGEL). Equal amounts of RNA were retro-transcribed using RevertAid First Strand cDNA Synthesis Kits

(Fermentas), and real-time reverse transcription-PCR experiments were performed using SYBR Green (Bio-Rad) and a Bio-Rad machine. Primers used in this paper are available on request.

#### *Statistical analysis*

Student's *t*-test was used for statistical analysis and $P<0.05$ was considered to be statistically significant.

#### *Adipocyte differentiation and Oil Red O staining*

To induce adipocyte differentiation, C3H10T1/2 cells transduced with indicated viruses were pre-treated with 100 ng/μl BMP7 in DMEM supplemented with 10% FBS for 3 days to reach confluence (commit to pre-adipocytes), followed by 2-day treatment with 0.5 μM dexamethasone (D1756, Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, I5879, Sigma), 20 nM insulin, 0.125 mM indomethacin, and 1 nM triiodothyronine (T3) in DMEM supplemented with 10% FBS, and then switched to DMEM with 10% FBS supplemented with 20 nM insulin and 1 nM triiodothyronine (T3). Medium was changed every 2 days until the cells were harvested at the indicated time points.

For detection of lipid with Oil Red O, briefly, cells in 24-well plate were washed twice with PBS, fixed with 10% formaldehyde for 30–60 min, stained for 10 min at room temperature with Oil Red O solution (1.8 mg/ml Oil Red O in 60% isopropyl alcohol), and then washed with $H_2O$.

#### *Alkaline phosphatase staining assay*

For ALP staining assay, stable C2C12 cells expressing empty vector or UBE2O were seeded into 24-well plate, and when the cells reached confluence, 50 ng/ml BMP6 was added for another 3 days. Cells were fixed by 3.7% formaldehyde. Naphotol AS-MX phosphate (Sigma) and fast blue RR salt (Sigma) were added into the cells for histochemical examination of ALP activity.

#### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

## Acknowledgements

We thank Dr Karen Lyons for supplying *SMAD6*-knockout MEF cells, Midory Thorikay and Martijn JWE Rabelink for excellent technical assistance, and all other members in ten Dijke's lab for their kind help. This research was supported by Netherlands Research Council, Cancer Genomics Centre Netherlands, and Centre for Biomedical Genetics.

*Author Contributions:* XZ and PTD designed the experiments. AB and DWS performed the protein–protein interaction screen experiments. XZ and JZ performed the rest of experiments. XZ, JZ, and PTD wrote the manuscript. AB, DWS, CXL, and LZ provided research materials, helped with the experiments and provided valuable discussion.

## Conflict of interest

A Bauer, DW Selinger and CX Lu are employed by Novartis Institutes for Biomedical Research. The remaining authors declare that they have no conflict of interest.

## References

Al-Salihi MA, Herhaus L, Sapkota GP (2012) Regulation of the transforming growth factor β pathway by reversible ubiquitylation. *Open Biol* **2:** 120082

Bartke T, Pohl C, Pyrowolakis G, Jentsch S (2004) Dual role of BRUCE as an antiapoptotic IAP and a chimeric E2/E3 ubiquitin ligase. *Mol Cell* **14:** 801–811

Berleth ES, Pickart CM (1996) Mechanism of ubiquitin conjugating enzyme E2-230K: catalysis involving a thiol relay? *Biochemistry* **35:** 1664–1671

Burckstummer T, Bennett KL, Preradovic A, Schutze G, Hantschel O, Superti-Furga G, Bauch A (2006) An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. *Nat Methods* **3:** 1013–1019

Carracedo S, Lu N, Popova SN, Jonsson R, Eckes B, Gullberg D (2010) The fibroblast integrin α11β1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *J Biol Chem* **285:** 10434–10445

Chen D, Zhao M, Mundy GR (2004) Bone Morphogenetic Proteins. *Growth Factors* **22:** 233–241

Choi K-C, Lee YS, Lim S, Choi HK, Lee C-H, Lee EK, Hong S, Kim I-H, Kim S-J, Park SH (2006) Smad6 negatively regulates interleukin 1-receptor-Toll-like receptor signaling through direct interaction with the adaptor Pellino-1. *Nat Immunol* **7:** 1057–1065

Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, Martello G, Stinchfield MJ, Soligo S, Morsut L, Inui M, Moro S, Modena N, Argenton F, Newfeld SJ, Piccolo S (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFβ signaling, controls Smad4 monoubiquitination. *Cell* **136:** 123–135

Estrada KD, Retting KN, Chin AM, Lyons KM (2011) Smad6 is essential to limit BMP signaling during cartilage development. *J Bone Mineral Res* **26:** 2498–2510

Goto K, Kamiya Y, Imamura T, Miyazono K, Miyazawa K (2007) Selective inhibitory effects of Smad6 on bone morphogenetic protein type I receptors. *J Biol Chem* **282:** 20603–20611

Hagai T, Toth-Petroczy A, Azia A, Levy Y (2012) The origins and evolution of ubiquitination sites. *Mol Biosyst* **8:** 1865–1877

Haldeman MT, Finley D, Pickart CM (1995) Dynamics of ubiquitin conjugation during erythroid differentiation *in vitro*. *J Biol Chem* **270:** 9507–9516

Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K (1997) Smad6 inhibits signalling by the TGF-β superfamily. *Nature* **389:** 622–626

Inamitsu M, Itoh S, Hellman U, ten Dijke P, Kato M (2006) Methylation of Smad6 by protein arginine N-methyltransferase 1. *FEBS Lett* **580:** 6603–6611

Inui M, Manfrin A, Mamidi A, Martello G, Morsut L, Soligo S, Enzo E, Moro S, Polo S, Dupont S, Cordenonsi M, Piccolo S (2011) USP15 is a deubiquitylating enzyme for receptor-activated SMADs. *Nat Cell Biol* **13:** 1368–1375

Itoh S, ten Dijke P (2007) Negative regulation of TGF-β receptor/Smad signal transduction. *Curr Opin Cell Biol* **19:** 176–184

Kawabata M, Inoue H, Hanyu A, Imamura T, Miyazono K (1998) Smad proteins exist as monomers *in vivo* and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO J* **17:** 4056–4065

Klemperer NS, Berleth ES, Pickart CM (1989) A novel, arsenite-sensitive E2 of the ubiquitin pathway: purification and properties. *Biochemistry* **28:** 6035–6041

Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, Ebina M, Nukiwa T, Miyazawa K, Imamura T, Miyazono K (2003) Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7. *EMBO J* **22:** 6458–6470

Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K, Miyazawa K (2004) Negative regulation of transforming growth factor-beta (TGF-β) signaling by WW domain-containing protein 1 (WWP1). *Oncogene* **23:** 6914–6923

Lowery JW, de Caestecker MP (2010) BMP signaling in vascular development and disease. *Cytokine Growth Factor Rev* **21:** 287–298

Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL (1998) Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J Biol Chem* **273:** 25628–25636

Markson G, Kiel C, Hyde R, Brown S, Charalabous P, Bremm A, Semple J, Woodsmith J, Duley S, Salehi-Ashtiani K, Vidal M, Komander D, Serrano L, Lehner P, Sanderson CM (2009) Analysis of the human E2 ubiquitin conjugating enzyme protein interaction network. *Genome Res* **19:** 1905–1911

Miyazono K, Kamiya Y, Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. *J Biochem* **147:** 35–51

Murakami G, Watabe T, Takaoka K, Miyazono K, Imamura T (2003) Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol Biol Cell* **14:** 2809–2817

Neumann K, Endres M, Ringe J, Flath B, Manz R, Haupl T, Sittinger M, Kaps C (2007) BMP7 promotes adipogenic but not osteo-/chondrogenic differentiation of adult human bone marrow-derived stem cells in high-density micro-mass culture. *J Cell Biochem* **102:** 626–637

Nohe A, Keating E, Knaus P, Petersen NO (2004) Signal transduction of bone morphogenetic protein receptors. *Cell Signal* **16:** 291–299

Park SH (2005) Fine tuning and cross-talking of TGF-β signal by inhibitory Smads. *J Biochem Mol Biol* **38:** 9–16

Plouhinec JL, Zakin L, De Robertis EM (2011) Systems control of BMP morphogen flow in vertebrate embryos. *Curr Opin Genet Dev* **21:** 696–703

Ruschke K, Hiepen C, Becker J, Knaus P (2012) BMPs are mediators in tissue crosstalk of the regenerating musculoskeletal system. *Cell Tissue Res* **347:** 521–544

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68:** 850–858

Sieber C, Kopf J, Hiepen C, Knaus P (2009) Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev* **20:** 343–355

Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T (2011) Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* **27:** 431–432

Suenaga M, Matsui T, Funaba M (2010) BMP Inhibition with dorsomorphin limits adipogenic potential of preadipocytes. *J Vet Med Sci* **72:** 373–377

Tang LY, Zhang YE (2011) Non-degradative ubiquitination in Smad-dependent TGF-β signaling. *Cell Biosci* **1:** 43

Tatham MH, Rodriguez MS, Xirodimas DP, Hay RT (2009) Detection of protein SUMOylation *in vivo*. *Nat Protoc* **4:** 1363–1371

Tokarev AA, Munguia J, Guatelli JC (2011) Serine-threonine ubiquitination mediates downregulation of BST-2/tetherin and relief of restricted virion release by HIV-1 Vpu. *J Virol* **85:** 51–63

Tseng Y-H, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, Tran TT, Suzuki R, Espinoza DO, Yamamoto Y, Ahrens MJ, Dudley AT, Norris AW, Kulkarni RN, Kahn CR (2008) New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* **454:** 1000–1004

Wang X, Herr RA, Chua WJ, Lybarger L, Wiertz EJ, Hansen TH (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J Cell Biol* **177:** 613–624

Wefes I, Mastrandrea LD, Haldeman M, Koury ST, Tamburlin J, Pickart CM, Finley D (1995) Induction of ubiquitin-conjugating enzymes during terminal erythroid differentiation. *Proc Natl Acad Sci USA* **92:** 4982–4986

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. *Science* **242:** 1528–1534

Wu MY, Hill CS (2009) Tgf-β superfamily signaling in embryonic development and homeostasis. *Dev Cell* **16:** 329–343

Xie Z, Chen Y, Li Z, Bai G, Zhu Y, Yan R, Tan F, Chen YG, Guillemot F, Li L, Jing N (2011) Smad6 promotes neuronal differentiation in the intermediate zone of the dorsal neural tube by inhibition of the Wnt/β-catenin pathway. *Proc Natl Acad Sci USA* **108:** 12119–12124

Yokota T, Nagai H, Harada H, Mine N, Terada Y, Fujiwara H, Yabe A, Miyazaki K, Emi M (2001) Identification, tissue expression, and chromosomal position of a novel gene encoding human ubiquitin-conjugating enzyme E2-230k. *Gene* **267:** 95–100

Zhang J, Zhang X, Zhang L, Zhou F, van Dinther M, Ten Dijke P (2012a) LRP8 mediates Wnt/β-catenin signaling and controls osteoblast differentiation. *J Bone Miner Res* **27:** 2065–2074

Zhang L, Huang H, Zhou F, Schimmel J, Pardo CG, Zhang T, Barakat TS, Sheppard KA, Mickanin C, Porter JA, Vertegaal AC, van Dam H, Gribnau J, Lu CX, Ten Dijke P (2012b) RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad7 for degradation. *Mol Cell* **46:** 650–661

Zhang X, Zhang J, Zhang L, van Dam H, Ten Dijke P (2013) UBE2O negatively regulates TRAF6-mediated NF-κB activation by inhibiting TRAF6 polyubiquitination. *Cell Res* **23:** 366–377

Zhou F, Zhang X, van Dam H, Ten Dijke P, Huang H, Zhang L (2012) Ubiquitin-specific protease 4 mitigates Toll-like/interleukin-1 receptor signaling and regulates innate immune activation. *J Biol Chem* **287:** 11002–11010