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Molecular mechanisms of novel regulators in cytokine signal transduction

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Chapter 2

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

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Abstract

SMAD6 is a crucial feedback inhibitory regulator of bone morphogenetic protein (BMP)/SMAD Signaling. 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 Signaling in a SMAD6-dependent manner. Addressing the molecular mechanism by which UBE2O and monoubiquitinated SMAD6 potentiate BMP7 Signaling, 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.

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 signaling has been demonstrated (Chen et al, 2004; Lowery & de Caestecker, 2010; Miyazono et al, 2010; Ruschke et al, 2012; Sieber et al, 2009; Wozney et al, 1988). BMPs have been implicated in embryogenesis and development through their controlling of cell proliferation, differentiation, and apoptosis (Miyazono et al, 2010; Nohe et al, 2004; 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 signaling 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 & Hill, 2009). One of the BMP signaling target genes is inhibitory (I-)SMAD6, has been shown to negatively regulate the BMP signaling (Itoh & 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- κ B 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 & 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.

Materials and Methods

Tandem affinity purification (TAP)

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 x 2 contingency table was constructed describing the number of purifications in which the interactor was observed with the bait vs. 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. Non-ribosomal proteins (ribosomal proteins are also found in many other "bait proteins" interactome) with p-values ≤ 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, immortalized wild-type or SMAD6 knockout MEFs (Estrada et al, 2011) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 U/ml penicillin/streptomycin (Invitrogen). SV40 retroviruses were used to immortalize 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-K174 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 materials and methods. 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 rpm 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 rpm 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 Materials and methods.

Protein purification and in vitro interaction

To obtain purified monoubiquitinated SMAD6 (Flag-Ubi-SMAD6), Flag-SMAD6, 10 $\times$ 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 μ g/ml Flag peptide (Sigma) in *in vitro* binding buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 0.1% NP40, 5% glycerol). Flag peptides were discarded, and eluates were concentrated with 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 by Amicon Ultra 3K (Millipore).

For *in vitro* protein-protein interactions, purified Flag-SMAD6 or F-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 μ M, 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 MgCl₂, 1 mM DTT, 2 mM ATP). A total of 45 μ l of the 50 μ l reaction system was diluted with RIPA buffer and followed with anti-Flag resin immunoprecipitation. A total of 5 μ l of the 50 μ l 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 rpm 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 Na₂HPO₄, 0.1 M NaH₂PO₄, 10 mM Tris-HCl, 10 mM imidazole, 10 mM β -mercaptoethanol). Lysates were centrifuged at 4,000 rpm for 10 min at room temperature and incubated with nickel beads at RT 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/ μ l BMP7 in DMEM supplemented with 10% FBS for 3 d to reach confluence (commit to preadipocytes), followed by 2 d treatment with 0.5 μ M 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

30

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 d 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 H₂O.

Results

Identification of UBE2O as an interacting protein of SMAD6

Figure 1

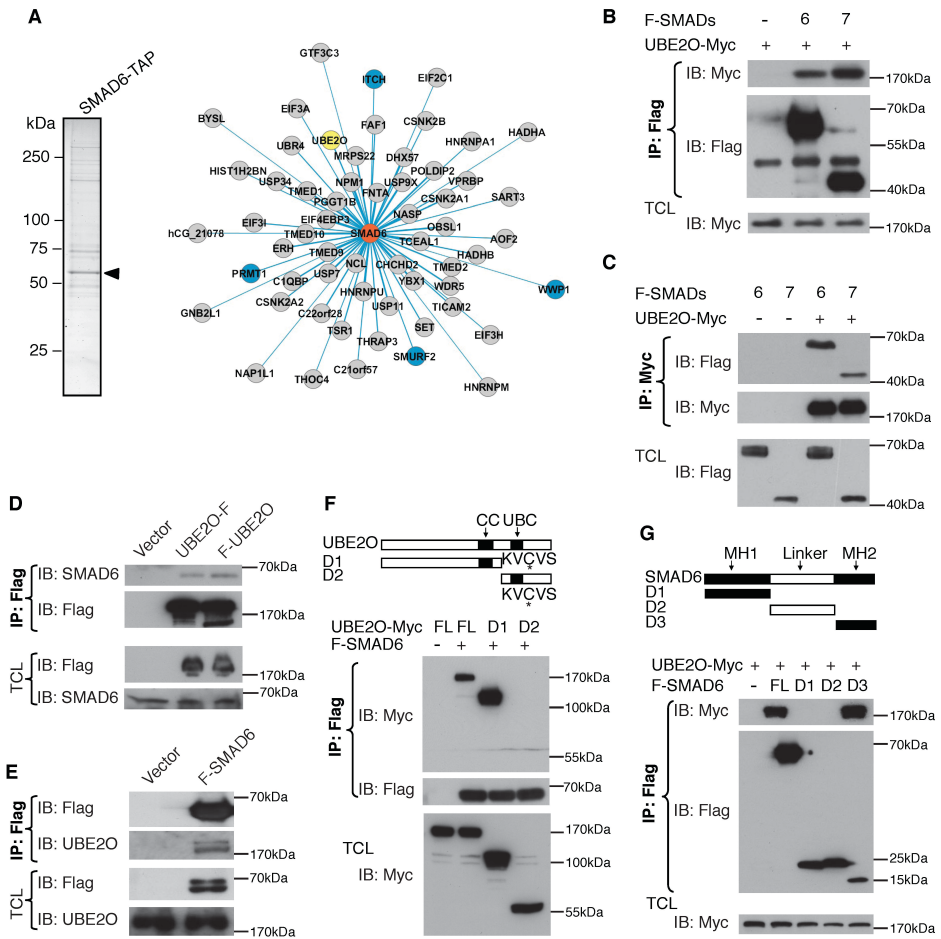


Figure 1 Identification of UBE2O as a SMAD6 interacting partner. (A) Proteomic screening of interacting protein for SMAD6. HEK293T cells transfected with TAP-SMAD6 were lysed for immunoprecipitation. Purified protein complexes were separated by SDS-PAGE and stained by colloidal Coomassie Blue. Triangle indicates accumulation of SMAD6. Blue or gray 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) and (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) and (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.

SMAD6 and SMAD7 play key roles in inhibiting BMPs and/or TGF- β -mediated signaling (Itoh & ten Dijke, 2007; Park, 2005). To pinpoint novel regulators of BMP/TGF- β signaling 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 and unpublished observations), and several known interacting proteins for SMAD6 and/or SMAD7 were confirmed in our screen (blue ovals in Figure 1A) (Al-Salihi et al, 2012; Inamitsu et al, 2006; Komuro et al, 2004; Murakami et al, 2003). 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). 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 1C). We next focused on UBE2O in SMAD6-mediated signaling 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 1E). 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 1G). 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 & 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 mediating the monoubiquitination of SMAD6.

Figure 2

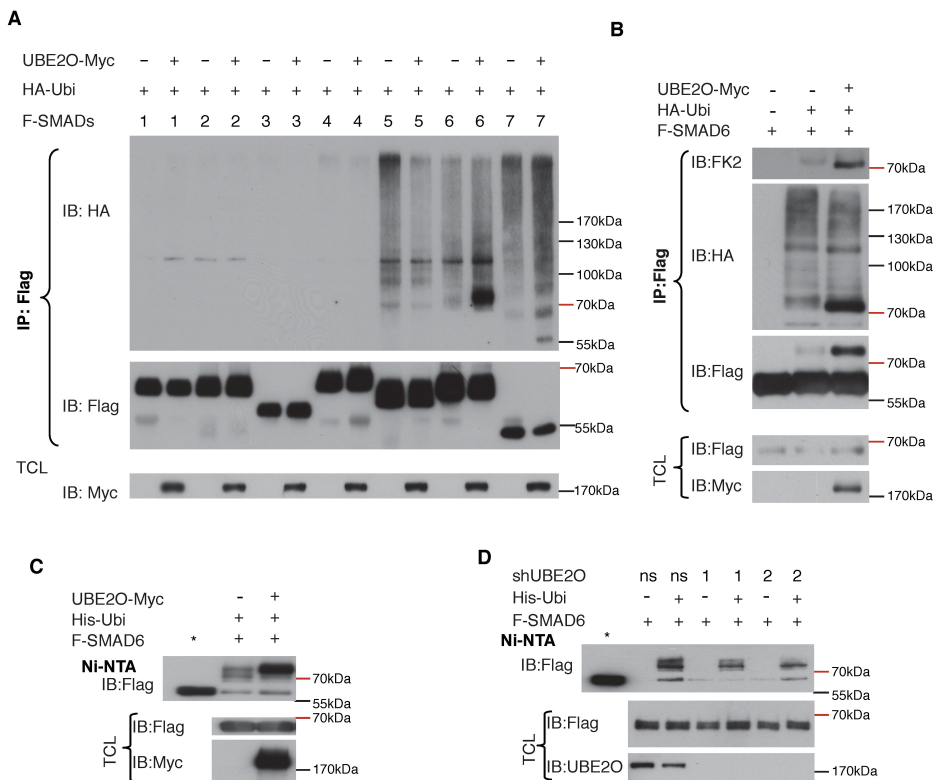


Figure 2 UBE2O monoubiquitinates SMAD6. (A) UBE2O specifically ubiquitinates SMAD6 and SMAD7. HEK293T cells were transfected with indicated Flag-SMADs and HA-ubiquitin with or without UBE2O-Myc for 48 h. *In vivo* ubiquitination of SMADs proteins was performed by immunoprecipitation. Cells were sonicated with 1% SDS, then diluted to 0.1% SDS for immunoprecipitation with anti-Flag resin. HA antibody was used to detect ubiquitinated SMADs. (B) and (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 Figure 2A. Monoubiquitinated SMAD6 was detected by HA or FK2 (specifically recognize 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 (shUBE2O-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.

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* (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 (Figure S1B). These results suggest that UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6.

Figure 3

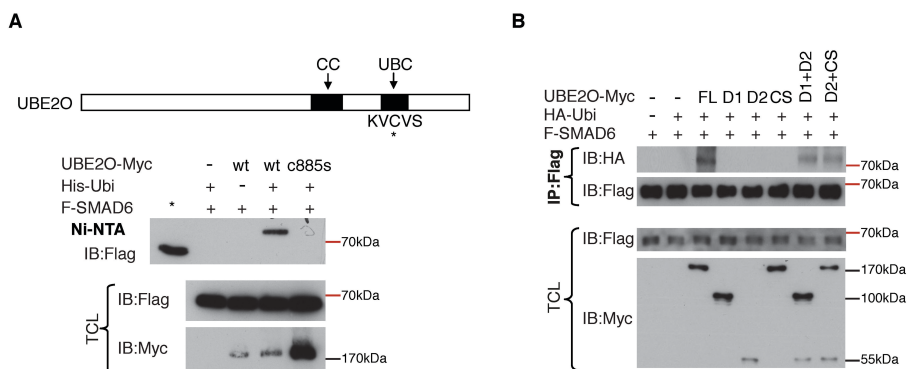


Figure 3 UBE2O functions as an E2-E3 hybrid to monoubiquitinate SMAD6. (A) Cysteine 885 is the E2 active site of UBE2O for monoubiquitinating SMAD6. The conserved and putative E2 active site cysteine 885, located in the ubiquitin-conjugating domain of UBE2O, was mutated to serine. HEK293T cells were 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, and UBE2O deletions (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.

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 monoubiquitinated 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 4C). 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.

Figure 4

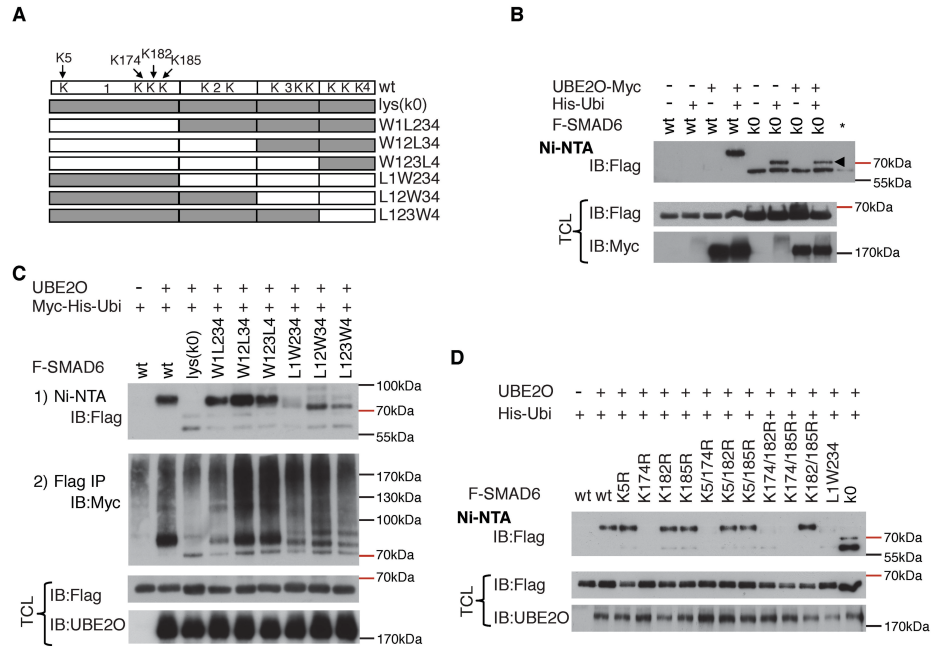


Figure 4 Lysine 174 of SMAD6 is monoubiquitinated by UBE2O. (A) Schematic of the chimeric SMAD6. Wild-type (wt) and lysine deficient (k0) SMAD6 were used to make chimeric SMAD6. SMAD6 was divided into 4 regions and chimeric SMAD6 was named according to presence of lysine. Example: W1L234 was made by 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 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 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 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.

Monoubiquitination of SMAD6 enhances BMP7-induced signaling

To investigate the function of monoubiquitinated SMAD6, we screened all known reported signaling pathways that could be affected by SMAD6 (Figure S2A-S2G). Of these signaling 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 (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 S3B and S3C); this increase in UBE2O expression levels 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 S3B and S3C). Taken together, our findings suggest that UBE2O monoubiquitinates SMAD6 to attenuate its inhibitory effects and thus augments BMP7-induced signaling in adipocyte differentiation.

Figure 5

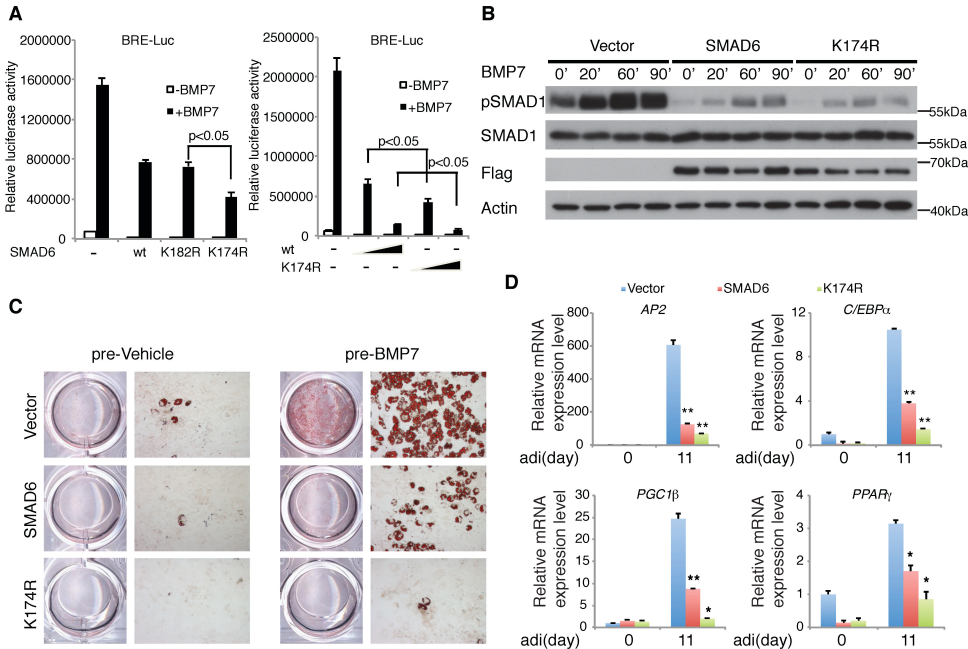


Figure 5 Monoubiquitination of SMAD6 potentiates BMP7-induced signaling. (A) Lysine 174-mutated SMAD6 increases the inhibitory ability of SMAD6 on BMP7-induced signaling. Analysis of BMP7-induced signaling activation in HEPG2 cells with co-transfection of SMAD6 (wt), lysine 182 or 174 to arginine (K182R or K174R, left panel) or increase amounts of lysine 174 to arginine (K174R, right panel). Data from triplicates are presented as the mean $\pm$ SD 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 analyze 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 material 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* , *PGC1 β* , and *PPAR γ* were analyzed 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).

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

Figure 6

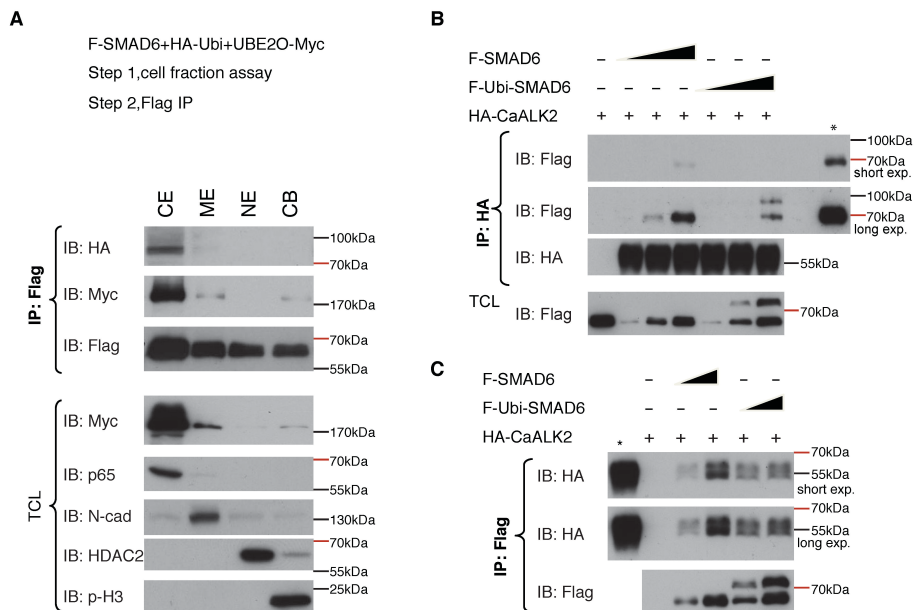


Figure 6 Monoubiquitinated SMAD6 restrains the interaction of ALK2 and SMAD6. (A) Subcellular localization of monoubiquitinated SMAD6. HEK293T cells were transfected with Flag-SMAD6, HA-ubiquitin, and UBE2O-Myc for 48 h. Cell fractionation assays were performed as described in material and methods. Four 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) and (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 analyzed with indicated antibodies. Asterisks indicate the input.

BMP7 activates signaling primarily via ALK2 (Macias-Silva et al, 1998), and co-transfection with SMAD6 resulted in a strong interaction with constitutively active ALK2 (Figure S4A; (Goto et al, 2007)). We also found that monoubiquitination-defective

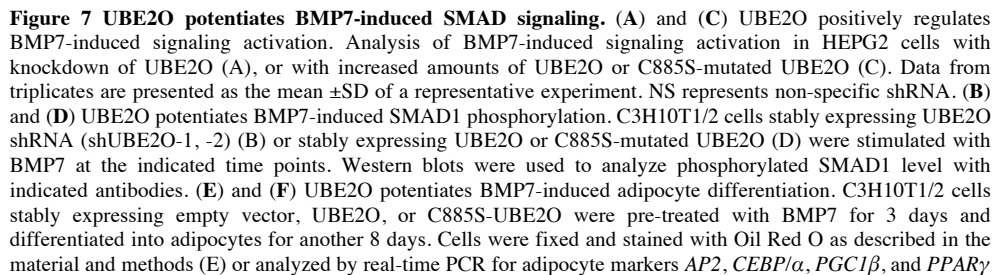
SMAD6 was more potent at inhibiting BMP7-induced SMAD1 phosphorylation (Figure 5B). In addition, analysis of the subcellular distribution of (monoubiquitinated) SMAD6 protein via cell fractionation demonstrated that monoubiquitinated SMAD6 (like UBE2O) was predominantly localised in the cytoplasm, whereas non-modified SMAD6 protein was present in all subcellular fractions (Figure 6A and Figure S4B). Moreover, in agreement with this latter finding, an artificial SMAD6-ubiquitin fusion protein was absent from the nucleus and present in the cytoplasm (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). 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 6C). 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 signaling

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 7B). 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-7F).

To confirm that the effect of UBE2O on BMP7 signaling occurs via alternation of SMAD6 function, we created a UBE2O-overexpressing stable cell line in SMAD6-knockout fibroblast cells (Figure S5). In line with the antagonistic function of SMAD6 in BMP signaling (Estrada et al, 2011), BMP7-induced SMAD1 phosphorylation was potentiated in *SMAD6*-knockout cells (Figure 7G and 7H). Notably, UBE2O increased BMP7-induced SMAD1 phosphorylation levels only in wild-type fibroblast cells and not in *SMAD6*-knockout fibroblast cells (Figure 7G and 7H). Thus, UBE2O potentiates BMP7-induced signaling, which depends at least in part on SMAD6 expression.

A



(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) The potentiated BMP7-induced signaling by UBE2O depends on SMAD6. Immortalized SMAD6 knockout mouse embryonic fibroblast (MEF) cells were infected with empty vector or UBE2O-expression viruses. Cells were treated with BMP7 at the indicated time points. Phosphorylated SMAD1 level was analyzed by western blot. (H) The relative BMP7 induced SMAD1 phosphorylation level in Figure 7G (normalized to SMAD1) was quantified.

Discussion

SMAD6 is a critical feedback regulator that limits BMP signaling, 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 signaling (Dupont et al, 2009; Inui et al, 2011). Unlike the polyubiquitination associated with protein degradation, 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 signaling and BMP7-induced adipocyte differentiation (Figure 8).

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 monoubiquitinates 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 (Hagai et al, 2012; Tokarev et al, 2011; Wang et al, 2007). 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 monoubiquitinate SMAC (Bartke et al, 2004). UBE2O has also been suggested to function as an E2-E3 hybrid (Berleth & 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.

Figure 8

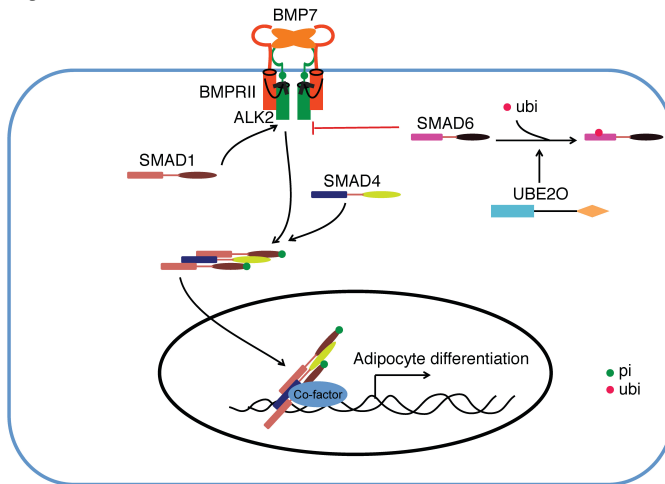


Figure 8 Schematic shows how UBE2O potentiates BMP7 signaling. Without monoubiquitination, SMAD6 binds to activated type I receptor to inhibit BMP7-induced SMAD1 phosphorylation and subsequent regulation of transcriptional responses. Monoubiquitinated SMAD6 by UBE2O impairs the association of activated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

The function of monoubiquitinated SMAD6 and UBE2O in BMP7 signaling

SMAD6 plays an inhibitory role in several signal pathways, such as the TGF- β /BMPs, Wnts, and IL-1R pathways (Choi et al, 2006; Imamura et al, 1997; Xie et al, 2011). However, monoubiquitination-disabled SMAD6 exerts a significantly more potent antagonising effect on BMP7-induced signaling and SMAD1 activation, which suggests that monoubiquitinated SMAD6 specifically exerts weaker inhibitory activity on BMP7 signaling 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 signaling.

In addition, we showed that the positive effect of UBE2O on BMP7 signaling was SMAD6-dependent, as UBE2O failed to potentiate BMP7-induced SMAD1 phosphorylation when SMAD6 was depleted. Although the UBE2O-mediated potentiation of BMP7-induced signaling 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) and 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 (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 signaling, at least in part, via SMAD6 monoubiquitination.

Monoubiquitinated 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 & 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 signaling.

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 signaling 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. BMP signaling was also shown to be 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 signaling in pre-adipocyte commitment and adipocyte differentiation. In addition, we observed that UBE2O misexpression in C2C12 myoblast cells also affected BMP-induced SMAD1 phosphorylation and expression of an early osteoblast maker, i.e., alkaline phosphatase activity (unpublished observations). In conclusion, we identified UBE2O as a pivotal positive regulator of BMP7 signaling in mesenchymal stem cells.

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Author Contribution

XZ and PTD designed the experiments. AB, DW performed the protein-protein interaction screen experiments. XZ and JZ performed the rest of experiments. XZ, JZ and PTD wrote the manuscript. AB, DW, CXL and LZ provided research materials, helped with the experiments and provided valuable discussion.

Conflict of Interest

The authors declare that they have no conflict of interests. A. Bauer, D.W. Selinger and C.X. Lu are employed by Novartis Institutes for Biomedical Research.

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Supplementary Information

Supplementary Materials and Methods

Plasmids

Human UBE2O (Yokota et al., 2001) cDNA obtained from mRNA isolated from HEK293T cells was cloned into pCR3.1-Myc/Flag, pLV-bc-CMV-puro (pLV) lentivirus vector or modified pLV-Myc/Flag. Deletions of UBE2O D1 (1-732 amino acids (aa)) and D2 (732-1138 aa) were cloned into pLV-Myc/Flag. Mouse SMAD1 to SMAD7 was cloned into Flag-pcDNA3 vector. Deletions of SMAD6 D1 (1-180 aa), D2 (180-331 aa) and D3 (331-496 aa) were cloned into modified Flag-pLV. HA/His/Myc-His ubiquitin, HA-ALK2/3/6, HA tagged constitutively activated ALK2/3/6 (HA-caALK2/3/6), Flag-TRAF6, β -catenin, TGF- β /SMAD-responsive transcriptional reporter construct CAGA-Luc, Wnt/ β -catenin-responsive transcriptional reporter construct TOP-flash, NF- κ B-responsive transcriptional reporter construct IgK-Luc and BMP/SMAD-responsive transcriptional reporter construct BRE-Luc have been described previously (Korchynskyi and ten Dijke, 2002; Nakao et al., 1997; Zhang et al., 2012; Zhou et al., 2012). Human ubiquitin was ligated at carboxy (C) terminus of SMAD6 to construct Flag-SMAD6-ubiquitin fused encoding plasmid. All new constructs were confirmed by DNA sequencing.

Transfections and stable cell line selection

HEPG2 and HEK293T cells were transfected with polyethylenimine (PEI, Sigma). For the luciferase assay, HEPG2 or HEK293T cells seeded in a 24-well plate were transfected with the indicated plasmids. Cells were harvested 36 h after transfection or 24 h after transfection followed by 12 h stimulation with BMP7 (50 ng/ml) or indicated ligands. Luciferase activity was measured using the luciferase reporter assay system from Promega and a Perkin Elmer luminometer. *LacZ* expression plasmid (25 ng/well) was cotransfected and used for normalization to control for differences in transfection efficiency. Each transfection was compensated with empty vector so that equal amounts of DNA were transfected, and every experiment was performed in triplicate. Every experiment was performed at least three times and representative results are shown. For the ubiquitination assay transfection, HEK293T cells seeded in 100-mm plates were transfected with the indicated plasmids using PEI.

Lentiviruses were produced by transfecting the indicated plasmids together with helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV into HEK293T cells as described before (Zhang et al., 2012). Cell supernatants were harvested at 48 h post transfection. To obtain stable cell lines, cells were infected for 24 h with lentivirus supernatant in the presence of 5 μ g/ml of polybrene (Sigma). After infection, cells were re-seeded into 6-well plates for western blotting, quantitative real-time PCR or adipocyte differentiation experiments under puromycin selection (C3H10T1/2, 4 μ g/ml; MEF cells, 4 μ g/ml). To knockdown UBE2O, Lentiviral shRNAs obtained from Sigma (MISSION® shRNA) were used. We tested five shRNAs target UBE2O from the mission library, and

two shRNAs that demonstrated efficient knockdown were chosen for further experiments. For human UBE2O, TRCN0000004587 (sh1) and TRCN0000004589 (sh2) were used. For mouse UBE2O, TRCN0000095042 (sh1) and TRCN0000095041 (sh2) were used in this study.

Immunofluorescence

HeLa cells transfected with Flag-SMAD6 or Flag-SMAD6-ubiquitin were fixed in 4% paraformaldehyde, washed with phosphate buffered saline (PBS), and blocked for 60 min with 10% fetal bovine serum (FBS) in PBS containing 0.1% Triton X-100. Cells were stained with anti-Flag antibody (Sigma), and then stained with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, USA). The images were acquired by a Leica confocal system.

Supplementary References

Korchynskyi, O., and ten Dijke, P. (2002). Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 277, 4883-4891.

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

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Figure S2

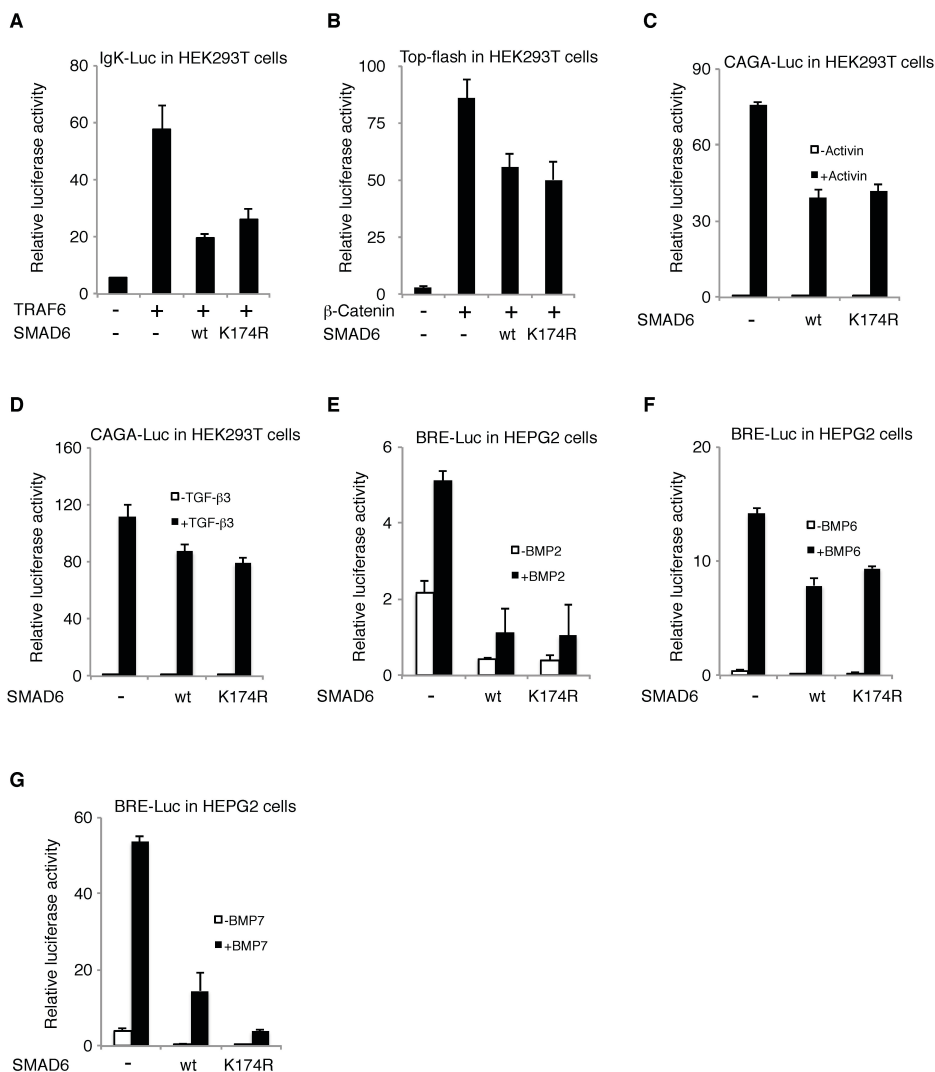


Figure S2 Lysine 174 mutation specifically increases SMAD6 inhibition effect on BMP7-induced signaling activation. (A)-(G) HEK293T or HEPG2 cells transfected with the TRAF6-dependent NF- κ B, Wnt/ β -catenin, TGF- β /SMAD or BMP/SMAD transcriptional reporter and SMAD6 or K174R SMAD6 expression plasmids in the absence or presence of appropriate stimuli and were subsequently analyzed for luciferase activity. Data from triplicates are presented as the mean $\pm$ SD of a representative experiment.

Figure S3

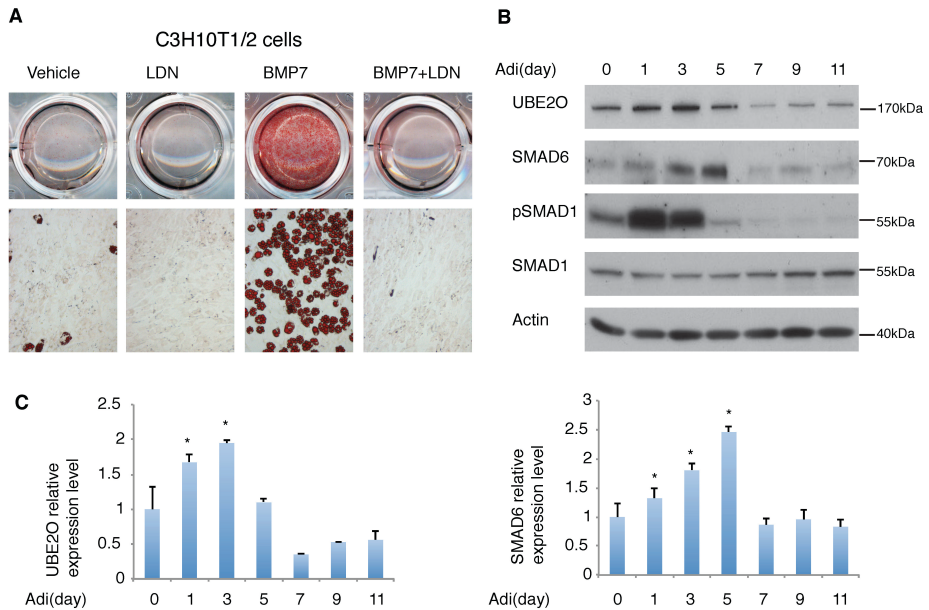


Figure S3 BMP7 induces adipocyte differentiation and upregulates UBE2O protein level. (A) BMP7-induced adipocyte differentiation is inhibited by BMP type I receptor inhibitor LDN-193189 (LDN). C3H10T1/2 cells were pretreated with the indicated reagents, induced to adipocytes as described in material and methods, and stained with Oil Red O. Whole well and higher magnification are shown at top and bottom panel, respectively. (B) SMAD6 and UBE2O are upregulated in BMP7 induces commitment of mesenchymal stem cells to proliferating preadipocytes. C3H10T1/2 cells were pre-treated with 100 ng/ μ l BMP7 in DMEM supplemented with 10% FBS for 3 d 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. (C) The relative protein level of UBE2O or SMAD6 (compared with protein level of UBE2O or SMAD6 without BMP7 treatment) was quantified. * indicates $p < 0.05$.

Figure S4

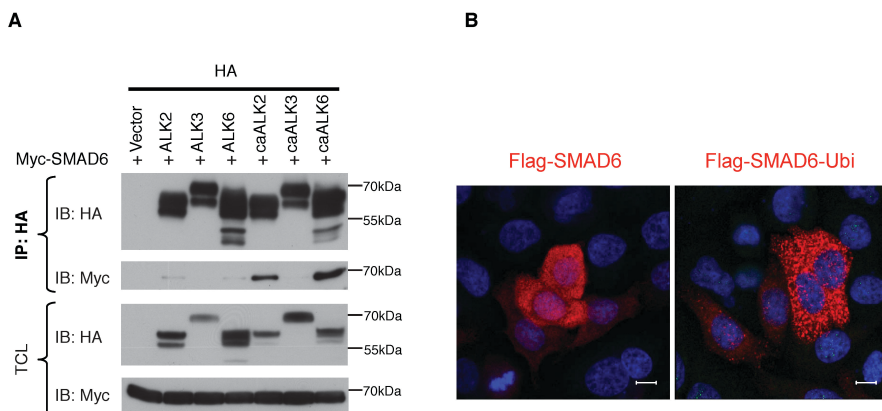


Figure S4 Interaction of SMAD6 and BMP Type I receptor and localisation of SMAD6-ubiquitin. (A) SMAD6 interacts with activated ALK2. HEK293T cells were transfected with indicated HA-tagged wild-type or constitutively active (ca) BMP type I receptors (i.e. ALK2, -3 and -6), and Myc-SMAD6. Immunoprecipitation with anti-HA resin was used to identify the receptor-associated SMAD6. (B) SMAD6-ubiquitin localises in the cytoplasm. HeLa cells transfected with Flag-SMAD6 or Flag-SMAD6-ubiquitin (Flag-SMAD6-Ubi) were immunostained with Flag antibody. Alexa Fluor 594 goat anti-mouse IgG was used to visualize the localisation of the Flag-tagged protein.

Figure S5

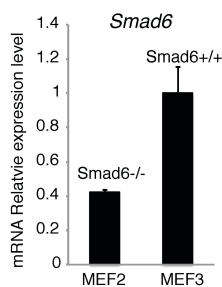


Figure S5 Validation of SMAD6 knockout MEF cells. Real-time PCR was used to detect the *SMAD6* mRNA level in *SMAD6* wild-type (+/+) or knockout (-/-) mouse embryonic fibroblast (MEF) cells.

Figure S6

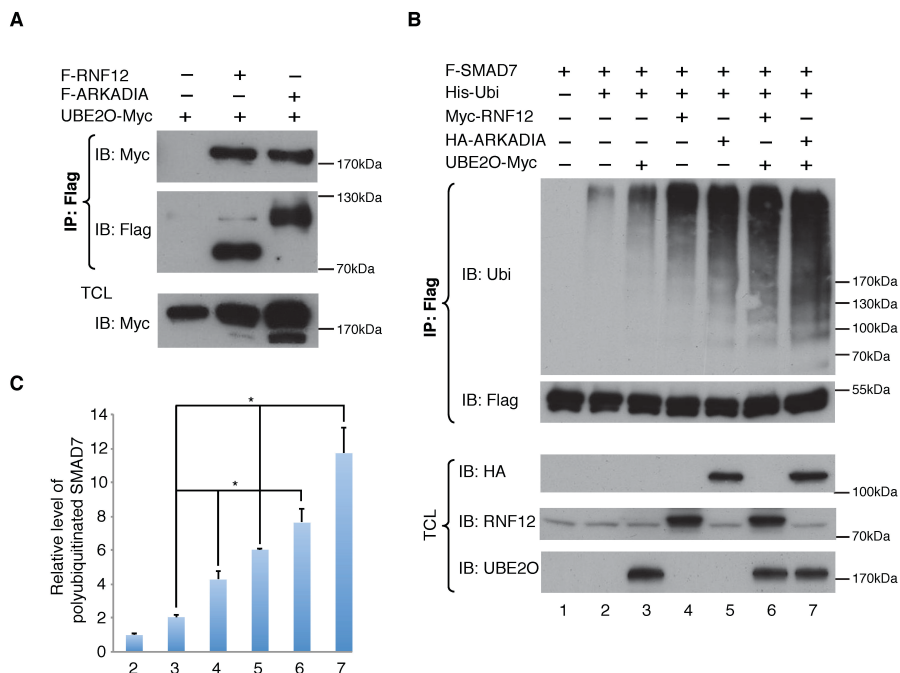


Figure S6 UBE2O potentiates RNF12 and ARKADIA mediated SMAD7 polyubiquitination. (A) HEK293T cells were transfected with UBE2O-Myc, Flag-RNF12 or Flag-ARKADIA as indicated for 48 h. Cells were harvested for immunoprecipitation with anti-Flag resin. Myc antibody was used to detect the interaction. (B) HEK293T cells were transfected with indicated plasmids for 48 h. Cells were treated with MG132 for 4 hours before harvesting. *In vivo* ubiquitination of SMAD7 was performed by immunoprecipitation. Cells were sonicated with 1% SDS, then diluted to 0.1% SDS for immunoprecipitation with anti-Flag resin. Polyubiquitinated SMAD7 was detected by ubiquitin antibody. (C) The relative polyubiquitinated SMAD7 level in B (polyubiquitinated SMAD7 by UBE2O and RNF12/ARKADIA was compared with UBE2O or RNF12/ARKADIA alone) was quantified. * indicates $p < 0.05$. IP, immunoprecipitation; IB, immunoblotting; TCL, total cell lysate.

Supplementary Table S1

IPI	Gene name	Max peptide count	Fisher p-value
IPI00012869.4	SMAD6	105	1.65E-26
IPI00217030.10	RPS4X	16	1.87E-15
IPI00604620.3	NCL	17	7.23E-13
IPI00007673.4	CHCHD2	6	2.32E-11
IPI00022759.2	PGGT1B	8	2.32E-11
IPI00023542.6	TMED9	5	2.32E-11
IPI00026813.1	FNTA	10	2.32E-11
IPI00217442.2	EIF4EBP3	11	2.32E-11
IPI00332499.1	NASP	14	2.32E-11
IPI00013485.3	RPS2	14	2.93E-11
IPI00549248.4	NPM1	15	8.00E-11
IPI00419880.6	RPS3A	20	3.67E-10
IPI00477802.1	TCEAL1	13	4.76E-10
IPI00644079.2	HNRNPU	10	1.10E-09
IPI00221012.7	USP9X	117	1.58E-09
IPI00028055.4	TMED10	7	4.10E-09
IPI00003965.4	USP7	6	8.91E-09
IPI00031812.3	YBX1	18	9.02E-09
IPI00472171.2	RPL7	10	3.58E-08
IPI00026271.5	RPS14	11	5.26E-08
IPI00184533.1	USP11	55	1.13E-07
IPI00021840.1	RPS6	7	1.13E-07
IPI00009976.1	TMED1	2	1.54E-07
IPI00306305.6	OBSL1	7	1.54E-07
IPI00165506.4	POLDIP2	4	1.54E-07
IPI00013146.1	MRPS22	3	1.99E-07
IPI00168885.5	DHX57	9	4.90E-07
IPI00005492.2	WDR5	7	7.79E-07
IPI00016613.2	CSNK2A1	11	7.79E-07
IPI00014230.1	C1QBP	105	1.59E-06
IPI00016608.1	TMED2	5	2.46E-06
IPI00029631.1	ERH	5	2.46E-06
IPI00221088.5	RPS9	12	2.98E-06
IPI00550689.3	C22orf28	6	4.27E-06
IPI00847579.1	RPS12	5	4.54E-06
IPI00216587.9	RPS8	12	7.44E-06

IPI00450975.1	RPS16	8	7.49E-06
IPI00783378.1	UBE2O	7	1.33E-05
IPI00292894.4	TSR1	6	1.40E-05
IPI00642513.2	RPS18_1_389	14	1.84E-05
IPI00072377.1	SET	7	3.48E-05
IPI00022793.4	HADHB	21	3.56E-05
IPI00070643.6	FAF1	2	3.67E-05
IPI00297593.4	USP34	9	3.67E-05
IPI00428967.2	TICAM2	4	3.67E-05
IPI00104050.3	THRAP3	5	3.67E-05
IPI00008530.1	RPLP0	12	5.32E-05
IPI00020602.1	CSNK2A2	6	6.58E-05
IPI00329528.10	VPRBP	10	6.58E-05
IPI00008529.1	RPLP2	7	7.18E-05
IPI00221089.5	RPS13	13	7.21E-05
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IPI00746934.2	UBR4	18	1.18E-04
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IPI00008433.4	RPS5	13	1.64E-04
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IPI00067923.4	C21orf57	2	5.14E-04
IPI00217540.7	AOF2	4	5.14E-04
IPI00215965.2	HNRNPA1	6	5.89E-04
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IPI00794461.1	HIST1H2BN	5	7.34E-04
IPI00412579.6	RPL10A	15	9.95E-04
IPI00006025.1	SART3	9	9.95E-04
IPI00221091.9	RPS15A	5	1.01E-03
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IPI00012750.3	RPS25	8	4.26E-03
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IPI00456758.4	RPL27A	6	4.30E-03
IPI00025091.3	RPS11	9	4.66E-03
IPI00023860.1	NAP1L1	2	5.53E-03
IPI00382885.1	RPL27	5	5.53E-03
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IPI00013009.1	WWP1	3	8.71E-03
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