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. Flag- and Myc-RNF12, 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-κB-responsive transcriptional reporter construct IgK-Luc and BMP/SMAD-responsive transcriptional reporter construct BRE-Luc have been described previously (Korchynskyi & ten Dijke, 2002; Nakao et al, 1997; Zhang et al, 2012a; Zhang et al, 2012b; Zhou et al, 2012). Flag- and Myc-ARKADIA was kindly given by Prof. Kohei Miyazono. 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 polyethyleneimine (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, 2012a). 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 µg/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 µg/ml; MEF cells, 4 µg/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, TRCN00000095042 (sh1) and TRCN00000095041 (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 Figure legends**

**Figure S1 Alignment of UBE2O and self-interaction of UBE2O**

(A) Alignment of the ubiquitin-conjugating domain of UBE2O in different species. The conserved putative E2 active site is conserved during evolution and cysteine that is essential for catalytic activity is indicated with an asterisk.

(B) Self-interaction of UBE2O. HEK293T cells were transfected with the indicated deletion mutant plasmids of UBE2O. Immunoprecipitation with anti-Flag resin was used to detect the self-interaction of UBE2O.

**Figure S2 Lysine 174 mutation specifically increases SMAD6 inhibition effect on BMP7-induced signalling activation.**

(A)-(G) HEK293T or HEPG2 cells transfected with the TRAF6-dependent NF-κB, 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 ±SD of a representative experiment.

**Figure S3 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.

**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 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 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, immunoprecipiation; IB, immunoblotting; TCL, total cell lysate.

**References**


Figure S1

A

UBE2O-Homo sapiens
UBE2O-Pan troglodytes
UBE2O-Macaca mulatta
UBE2O-Canis lupus
UBE2O-Bos taurus
UBE2O-Mus musculus
UBE2O-Rattus norvegicus
UBE2O-Gallus gallus
UBE2O-Drosophila melanogaster
UBE2O-Anopheles gambiae
UBE2O-Arabidopsis thaliana
UBE2O-Oryza sativa

B

UBE2O-D2-Flag
UBE2O-D1-Myc

- +
+ +

IB:Myc
IP:Flag
IB:Flag
TCL
IB:Myc

100kDa
70kDa
IgG-h
100kDa
Figure S2

A. IgK-Luc in HEK293T cells

B. Top-flash in HEK293T cells

C. CAGA-Luc in HEK293T cells

D. CAGA-Luc in HEK293T cells

E. BRE-Luc in HEPG2 cells

F. BRE-Luc in HEPG2 cells

G. BRE-Luc in HEPG2 cells
Figure S3

A

C3H10T1/2 cells

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<tr>
<th>Vehicle</th>
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<th>BMP7</th>
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[Images of cells under different conditions]
Figure S4

A

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<tr>
<th></th>
<th>+ Vector</th>
<th>+ ALK2</th>
<th>+ ALK3</th>
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70kDa  55kDa

TCL

IB: HA

70kDa

IB: Myc

70kDa

IB: HA

70kDa

IB: Myc

70kDa

B

Flag-SMAD6

Flag-SMAD6-Ubi
Figure S5

![Graph showing mRNA relative expression levels of Smad6 in MEF2 and MEF3 cells for Smad6+/+ and Smad6-/- genotypes.](image)