**Figure S1. VprBP negatively controls TGF-β signaling**

**A** Co-IP of the ectopically expressed ITCH-Myc and endogenous VprBP in HEK293T cells. Co-IP and input samples were analysed by immunoblotting. **B** Ectopic expression of VprBP has no effect on BRE-Luc BMP reporter activity in HEK293T cells with or without 50ng/ml BMP9 treatment for 16h. **C** Effect of VprBP knockdown on BMP-induced Smad1 and Smad2 phosphorylation, as measured by immunoblot analysis. HEK293T cells were treated with 50ng/ml BMP for 1 hour. **D** Effect of CUL4A and VprBP over-expression on CAGA-Luc TGF-β reporter activity in HEK293T cells, treated with or without 2.5ng/ml TGF-β for 16h. HEK293T cells were transfected with pcDNA3-Myc- CUL4A or pCMV-Myc-VprBP plus CAGA/BRE/ARE reporter and renilla plasmids. **E** Effect of ectopic VprBP expression on TGF-β target genes in MDA-MB-231 cells treated with or without 5ng/ml TGF-β for 6h. The mRNA levels of VprBP and target genes were determined by qPCR. *GAPDH* was used for normalization; mean ± SD of triplicates.
Figure S2. VprBP has no effect on the ubiquitination of R-Smads, Smad7 and Smurf2-Smad7 complex.

A Ubiquitination of Flag-Smad7 in HEK293T cells with or without VprBP overexpression. HEK293T cells were transfected with the indicated plasmids and treated with MG132 (5 mM). B Ubiquitination of Flag-Smad2 and 3 in HEK293T cells with or without VprBP overexpression. HEK293T cells were transfected with the indicated plasmids and treated with MG132 (5 mM). C Ubiquitination of Flag-Smad1 and 5 in HEK293T cells with or without VprBP overexpression. HEK293T cells were transfected with the indicated plasmids and treated with MG132 (5 mM). D qPCR analysis of mRNA levels of TβRI, Smad2, Smad3 and Smad4 as indicated in control or VprBP knockdown HaCaT cells. Normalized to GAPDH expression, mean ± SD of triplicates. E Co-IP of the ectopically expressed Myc-Smurf1 and TβRI-Flag in control or VprBP overexpression HEK293T cells. Co-IP and input samples were analysed by immunoblotting. F Co-IP of the ectopically expressed Flag-Smad7 and endogenous Smurf2 in control or VprBP knock down HEK293T cells. Co-IP and input samples were analysed by immunoblotting.
Figure S3. VprBP regulates Smurf1 stabilization.

A Top: Schematic presentation of VprBP FL and deletion constructs. Bottom: Co-IP of endogenous Smurf1 and Flag-VprBP deletion mutants transfected in HEK293T cells and detected by immunoblotting. B Stable HA-Ubiquitin expressing HEK293T cells were co-transfected with expression constructs for Flag-tagged Smurf2 (WT or CA mutant) and Myc-tagged VprBP as indicated and, treated with MG132 (5 µM) for 5h, cell lysates were
immunoprecipitated with Flag antibody followed by immunoblotting for HA (and Flag). Input samples were analysed with Myc antibody. C Co-IP of the ectopically expressed Flag-Smurf2-HECT domain and endogenous Smurf2 in control or VprBP overexpression HEK293T cells. Co-IP and input samples were analysed by immunoblotting.

Figure S4. VprBP suppresses Activin signaling.

A Activin A induced gene expression in control and VprBP depleted HepG2 cells upon 25 ng/ml treated for 1h. Expression was measured by real time quantitative PCR (qPCR). GAPDH was used for normalization; mean ± SD of triplicates. B qPCR analysis of Activin A (25 ng/ml) induced mesendoderm markers in mESCs stably expressing control shRNA (sh
Ctrl) or VprBP shRNA (sh VprBP). Normalized to GAPDH expression, mean ± SD of triplicates. 

CqPCR analysis of VprBP expression in control and VprBP shRNA knock down mESCs. Normalized to GAPDH expression, mean ± SD of triplicates.

Figure S5. Alignment of human and zebrafish VprBP protein sequences.

Identities between human and zebrafish VprBP are highlighted in yellow. The region that was used to generate antibody against human VprBP is indicated by a red line.
Appendix Supplementary Methods

Plasmids

The pCMV-Myc-VprBP plasmid was kindly provided by Dr. Éric A. Cohen (Institut de Recherches Cliniques de Montre´al, Montre´al, Canada), and pLV-VprBP was sub-cloned from it and verified by sequencing. Flag-Smurf1, Smurf1 C699A, and Smurf1 deletions were provided by Dr. Hongrui Wang (Xiamen University, Xiamen, China). The VprBP deletions were gifts from Dr. Jun Huang (Zhejiang University, Hangzhou, China). The pcDNA3-Myc-CUL4A plasmid was provided by Dr. Yue Xiong (University of North Carolina at Chapel Hill, Chapel Hill, NC, USA)

Immunoblotting and immunoprecipitation

Cells were lysed using TNE buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 1% NP-40, and protease inhibitors) at 4°C. The lysate supernatants were obtained by a 10 min centrifugation (12,000 rpm) at 4°C. After measurement of protein concentration, equal amounts of protein were prepared for western blotting. To perform immunoprecipitation assays, cell lysates were incubated with first antibodies and protein A-Sepharose (GE Healthcare BioSciences AB) for 3 h at 4°C. Then, precipitates were washed three times with TNE buffer, and immunoprecipitated proteins were eluted by sample buffer at 100°C for 5 min. All western blotting was performed using a mini gel running system from Bio-Rad. The primary antibodies used for immunoblotting and immunoprecipitation were against the following proteins: VprBP (A301-887A, BETHYL), Smad2/3 (610842, BD Transduction Laboratories), phospho-Smad2(Ser465/467) (#3101, Cell Signaling), SMAD7 (sc-7004, Santa Cruz Biotechnology), TβRI (sc-398, Santa Cruz Biotechnology), Smad4 (sc-7966, Santa Cruz Biotechnology), Smurf1 (sc-25510, Santa Cruz Biotechnology), Smurf2 (sc-25511, Santa Cruz Biotechnology), HA (1583816, Roche), Flag (F3165, Sigma), and β-actin
(A5441, Sigma). The mouse and rabbit IgG were purchased from Cell signaling. All the secondary antibodies used were from Sigma.

**Lentivirus production and stable cell line generation**

The shRNA constructs of VprBP control vectors were obtained from Sigma-Aldrich (MISSION®) (human shRNA TRCN0000129280 and TRCN0000129344, mouse shRNA TRCN0000251844) and the lentiviruses were produced as previous described (Li et al., 2016). To generate the stable cell lines, cells were seeded at 20% confluence and infected using lentiviral supernatants (120 ng/ml of virus) containing of 8 ng/ml polybrene (Sigma) that were diluted in normal culture medium. Forty-eight hours after infection, cells were selected by puromycin for 5 days.

**RNA isolation and real-time quantitative RT–PCR**

The NucleoSpin RNA II kit (BIOKE) was used to isolate RNA and cDNA was obtained by reverse transcription-PCR (RevertAid First Strand cDNA Synthesis Kits, Fermentas). All real-time quantitative PCR (qPCR) was performed with the CFX Connect Real-Time PCR System (Bio-Rad) and calculated using CFX Manager software version 2.0 (Biorad). All mRNA expression levels were analysed in triplicate and normalized to GAPDH expression. All qPCR experiment was repeated 3 times. The primer sequences are listed in Appendix Supplementary Methods.

**RNA sequencing analysis**

Total RNA of mESCs was isolated and sequenced by Illumina-HiSeq2000/2500 platform. We used R package to get the expression of each gene by using all genes expressed FPKM. Genes were defined as differentially expressed genes displayed with a $p$ value $<0.01$. 
Embryonic stem cell culture and differentiation

The R1 mouse ES cell line was obtained from ATCC and cultured on 0.1% gelatin coated dishes in a monolayer in DMEM supplemented with 10% FBS, 1,000 U/ml LIF (Millipore), 1% nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma). Differentiation of mESCs in serum-free conditions is previously described (Ying and Smith, 2003). Briefly, the mESCs were cultured in a serum-free DMEM medium comprises N2 and B27 supplements (Giboco), 1% nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma). mESCs were treated with or without 25ng/ml Activin A during differentiation.

Zebrafish Embryo injection and in situ hybridization.

AB strain Zebrafish embryos were used in this assay. Whole-mount in situ hybridization was performed as described previously (Zhang et al., 2009). The morpholino oligonucleotides were synthesized by Gene Tools (vprbp MO 5’-AGACGCCATCACACCTTCAGCGAAC-3’ and control (ctrl) MO 5’-CCTCTTACCTCAGTTACAATTATA-3’). Morpholinos were injected into one-cell stage embryos and harvested at indicated stages. 2 ng vprbp MO or ctrl MO was injected per embryo. All zebrafish experiments were performed according to the guidelines for use of laboratory animals in Leiden University.

In vitro scratch assay

The MDA-MB-231 cells were plated in 24 well plates and grown to 70%-80% confluence as a monolayer. After 10 h serum starvation, two cross lines on cells were gently scratched using 200 µl pipette tips. Then, the detached cells were removed with new medium and treated with or without 5 ng/ml TGF-β for 36 h. The scratched monolayers were photographed at both 0 h and 36 h to analyse the relative migration.
Luciferase Reporter Assay

HEK293T cells were seeded in 24 wells plate and transfected with indicated plasmid plus CAGA/BRE/ARE reporter and renilla plasmids. At 24h after transfection, cells were treated with or without 2.5 ng/ml TGF-β, 50 ng/ml BMP9 or 25 ng/ml Activin A a for 16h. The luciferase activities were analysed by a luminometer (Berthold Technologies). Reporter activity was normalized to renilla expression.

cDNA cloning of zebrafish vprbp

The full-length zebrafish vprbp cDNA (encoding 1518 amino acids) was cloned from a zebrafish embryo cDNA library (24 hpf). This was done by nested polymerase chain reaction (PCR) using primers based on the sequence of Danio rerio VprBP-like sequences form NCBI (LOC108190994, LOC101884616, LOC100333146). The full coding sequence of zebrafish vprbp was amplified with first step primers (Forward: 5’-CATCAGAAAGTTGCAGCACATT-3'; reverse, 5'- CTTCATATCCATCATCCTTCTCTC-3') and second step primers (Forward: 5’-ATAGAATTCATGCGCTTCAGAGTGAGGC-3'; reverse, 5'- CGGATCGATTCACTCGTTCAGAGAGAAATG-3').

qPCR primers

The following human primers were used:

VprBP forward 5’-CTGCTATCTTGCTGTCATTG-3';
VprBP reverse 5’-CTCTTCATCTCCTCCTCTG-3';
GAPDH Forward: 5’-AGCCACATCGCTCAGACA C-3';
GAPDH Reverse: 5'-GCCCAATACGACCAAATC C-3';
PAI-1 Forward: 5’-GCAGGACATCCGGAGAGA-3';
PAI-1 Reverse: 5’-CTTGAGACACCTCCCTTGACCTT-3';
CTGF Forward: 5’-TGCGAAGCTGACCTGGAAGAA-3';
CTGF Reverse: 5’-AGCTCGGTATGTCTTCATGCTGCTGT-3';
IL-11 Forward: 5’-ACTGCTGCTGCTGAAGACTC-3';
IL-11 Reverse: 5’-CCACCCCTGCTCCTGAAATA-3';
PTHrP forward 5’-TTCTTCCCAGGTTGCTTGAG-3';
**PTHrP reverse** 5′-TTTACGGCGACGATTCTTCC-3′;

**Smad7 forward**, 5′-AGAGGCTGTGTGGCTGTAATC-3′;

**Smad7 reverse**, 5′-GCAGAGTCGGCTAAGGTGATG-3′;

**SnoN forward**, 5′-AAACTGAATGGGATGGGAGATG-3′;

**SnoN reverse**, 5′-TTTGCATGAATGTCCGTTATCAT-3′;

**p21 forward**, 5′-CCTCATCCGTTCTCTCTTT-3′;

**p21 reverse**, 5′-GTACCAACCAGGAGAACGTAATG-3′;

**p15 forward**, 5′-AAGCTGAGCCCAGGTCTCCTA-3′;

**p15 reverse**, 5′-CCACCGTTGAGCCGGTAAACT-3′.

**Mouse**

**GAPDH Forward**: 5′-AACCTTTGTCATTGTGGAGG-3′

**GAPDH Reverse**: 5′-TCACATTGGGGTGGAGGAACA-3′

**VprBP forward** 5′-GAGAATCAAGGCAGCATGGA-3′;

**VprBP reverse** 5′-CTTCCTGTGCCTCTCTTCA-3′;

**Oct4 forward**, 5′-CACGAGTGGAAAGCAACTCA-3′;

**Oct4 reverse**, 5′-AGATGGTGGTCTGGCTGAAC-3′;

**Sox3 forward**, 5′-CACAACTCCGAGACGAGCAA-3′;

**Sox3 reverse**, 5′-CTCGGGATTACTCTCTACTGTG-3′;

**T forward**, 5′-CATCGGAACAGCTCTCCAACCTAT-3′;

**T reverse**, 5′-GTGGGCTGGCGTTATGACTCA-3′;

**Pax6 forward**, 5′-GCAGATGGAACGCTCTCCAACCTAT-3′;

**Pax6 reverse**, 5′-CTTCCTGTGCCTCTCTTCA-3′;

**Lhx1 forward**, 5′-AAGCTCACCAGCTCACCAG-3′;

**Lhx1 reverse**, 5′-GCTGTCTCCTCTCCTCTTG-3′;

**Lefty1 forward**, 5′-CGCTGACCTCAAGGACTATG-3′;

**Lefty1 reverse**, 5′-ATCGGGTGCCTCTCAGTAC-3′;

**Lefty2 forward**, 5′-CCCGAGGCAAGAGGGTC-3′;

**Lefty2 reverse**, 5′-GTGAGTGGAGGTCTCTGACATCAG-3′;

**Gsc forward**, 5′-GTCAGAAAACGCCGAGAAGTG-3′;

**Gsc reverse**, 5′-GCTTTTACCTTCCTCTTCCCT-3′;

**Foa2 forward**, 5′-CCCGAGACTAAGCTCAGACGAT-3′;

**Foa2 reverse**, 5′-GCGTAGTGAGTCCTGCTGAGG-3′;

**Nanog forward**, 5′-GCAAGCGGTGGCAGAAAA-3′;

**Nanog reverse**, 5′-GTGCTGAGCCCTTCTGAATCA-3′.

**Zebrafish**

**vprbp forward** 5′-GGAGCAGCAGATGGAAGAG-3′;

**vprbp reverse** 5′-CGATGACGGCAAGGAGAAGTG-3′;

**gapdh forward** 5′-ATCTACTCCTTGGAGGAGCCATGT-3′;

**gapdh reverse** 5′-ATCTACTCCTTGGAGGAGCCATGT-3′
flh forward 5'- GAAGAGCTTACGAAACTTATGGAC-3'
flh reverse 5'-TACACAGCTCCACGACATGTTG-3'
gata5 forward 5'-CGACAACACTGTGGAGGAGA-3'
gata5 reverse 5'-GTAGCGCCAGACACTGTTGA-3'
mixer forward 5'-ACCCAAAGGAACACGAACAG-3'
mixer reverse 5'-AACAGGCGATGTGTNTAGGG-3'
chd forward 5'-AGACTGCTGTAAGGAGTGTCCCTC-3'
chd reverse 5'-CCATGAAGTCTCCTATGCATTCCG-3'
ntl forward 5'-ACCCATACACCCCAACCTC-3'
ntl reverse 5'-ATAATAGGACCGCTCATGC-3'
gsc forward 5'-GACGAGCAGCTGGAGGCACTGGA-3'
gsc reverse 5'-TCAGCTGTCAAGATCCACGTCGCT-3'
sox32 forward 5'-ACCTTGAATGAGACACACC-3'
sox32 reverse 5'-TGAAGGCAATTCTGTGCTCC-3'
sox17 forward 5'-CATGATGCCTGGCATGGGC-3'
sox17 reverse 5'-CACTCATACCTTCGTGCAC-3'
snail1a forward 5'-TCCTGCCCACACTGTAAAAG-3'
snail1a reverse 5'-GCGACTAAAGGTGCGAGAC-3'