

## Supplementary Data

### Supplementary methods

#### *Plasmids*

The following plasmids have been described: G5E4 and G0E4 (Lin et al., 1988), EF-HASmad4, ARE-luciferase and EFLacZ (Pierreux et al., 2000), EF-Flag-XFoxH1b (Howell et al., 2002), (Gal4-OP)<sub>5</sub>-luciferase (Randall et al., 2002) and pIC208G5E4 (Utley et al., 1998). For the ARE-E4, three ARE elements were cloned into G0E4. For Gal4 fused bacterial expression constructs, Gal4 (amino acids 1-95) was cloned into pET15b (Novagen) to generate 15b-Gal4. The FMSIM from *Xenopus* FoxH1b (amino acids 219-345), the C-terminal region of Smad2 (amino acids 198-467), the C-terminal region of Smad4 (amino acids 227-552) and full length Smad4 (amino acids 1-552) were cloned into 15b-Gal4. A G4-FMSIM mammalian expression construct was made by cloning G4-FMSIM into the pEF expression vector. For GST fused expression constructs human Smad4 or *Xenopus* Smad4 $\alpha$  and human Smad2 were cloned into pGEX-KG. For the preparation of phosphorylated Smad2, *Xenopus* Smad2 (amino acids 1-462) was cloned into pTXB1 (NEB).

#### *Recombinant and purified proteins*

Recombinant Gal4 fusion proteins were purified from bacteria by a two-step process, using Ni-NTA agarose (Qiagen) to isolate the His-tagged proteins and Heparin Sepharose (Amersham Biosciences). Recombinant unphosphorylated XSmad2 and hSmad4/XSmad4 $\alpha$  proteins were purified using glutathione agarose (Sigma), cleaving the GST moiety with thrombin. Phosphorylated XSmad2 was prepared using a peptide ligation strategy previously described (Cotton and Muir, 1999; Wu et al., 2001) and analyzed by mass spectrometry. Recombinant p300 was prepared as described (Kraus and Kadonaga, 1998). Recombinant Gal4-p53 (G4-p53) and Gal4-AH (which is a Gal4-fusion of 15 amino acid acidic peptide (Giniger and Ptashne, 1987) were gifts from Marc Timmers and Stefan Roberts respectively. Flag-XFoxH1b was purified by a Flag immunoprecipitation and elution with Flag peptide from NIH 3T3 transfected with Flag-XFoxH1b.

### ***Bandshift assays***

HaCaT nuclear extracts were prepared as previously described (Wong et al., 1999) from cells that were uninduced or induced with 2 ng/ml TGF- $\beta$  for 1 hour. The Gal4 bandshift probe used has been described (Randall et al., 2002). Bandshift assays with recombinant proteins (typically 50 ng of Gal4 fusion, 50 ng of unphosphorylated or Smad2P and 200 ng of Smad4) were incubated with 0.2 ng of Gal4 probe in binding buffer (15 mM Hepes pH 7.9, 100 mM KCl, 10  $\mu$ M ZnSO<sub>4</sub>, 7.5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.15 mM EDTA, 15% glycerol, 10  $\mu$ g HeLa nuclear extract, 10  $\mu$ g BSA and 1  $\mu$ g poly [d(I-C)]). Bandshift assays with endogenous proteins were performed as described (Germain et al., 2000) using 10  $\mu$ g of nuclear extract. For supershifts, 2  $\mu$ l of anti-Gal4 DBD (Santa Cruz), 1  $\mu$ l of anti-Smad2/3 (BD Biosciences), 1  $\mu$ l of anti-Smad4 (B8, Santa Cruz) or 1  $\mu$ l of anti-Flag (m2, Sigma) antibodies were added to the recombinant proteins or nuclear extract for 5 minutes before addition of probe.

### ***Immunoprecipitations***

For endogenous immunoprecipitations, HaCaT cells were either uninduced or induced with 2 ng/ml of TGF- $\beta$  for 1 hour and nuclear extracts prepared as described (Wong et al., 1999). Immunoprecipitations were performed essentially as previously described (Germain et al., 2000) and immunoprecipitates were fractionated on 10 or 15% SDS-polyacrylamide gels and Western blotted with anti-Smad2/3 (BD Biosciences), anti-Brg1 (Upstate) or anti-p300 (Pharmigen) antibodies as appropriate.

### ***Histone acetylation assays***

For histone acetylation assays, free HeLa core histones, recombinant histone octamers or dinucleosomes were incubated with recombinant proteins for 30 minutes at room temperature. Histone acetylation assays were performed at 30°C for 45 minutes in buffer containing 50 mM Tris pH 7.9, 100  $\mu$ M EDTA, 150 mM NaCl, 300  $\mu$ M ATP, 250  $\mu$ M creatine-phosphate, 16  $\mu$ M acetyl CoA and p300 or 30  $\mu$ g of HeLa nuclear extract. LysCoA was added at a final concentration of 1  $\mu$ M. Histones were fractionated on 18% SDS-polyacrylamide histone gels (Thomas and Kornberg, 1978) and Western blotted with anti-acetyl histone H3 (Upstate), anti-acetyl histone H4

(Serotec), anti-acetyl histone H3 K18, anti-acetyl histone H3 K9, anti-acetyl histone H4 K8 or anti-acetyl histone H4 K12 (Abcam) antibodies.

### ***siRNAs***

SMARTpool siRNA reagents were as follows: mouse Brg1 (catalogue number M-041135-00), mouse Brm (catalogue number M-056591-00), mouse Smad2 (catalogue number M-040707-00) and mouse p300 (catalogue number M-065607-00). Individual siRNA duplexes were as follows: mouse Brg1, Brg1 (1) (catalogue number D-041135-03) and Brg1 (3) (catalogue number D-041135-03). Control siRNAs used were RISC-Free siRNA (catalogue number D-001220-01-05) or non-targeting (catalogue number D-001206-13-05).

### ***ChIPs***

For ChIPs, P19 cells were treated overnight with 10  $\mu$ M SB-431542 to abolish autocrine signaling, before washing and inducing with 2 ng/ml Activin for 1 h. We used Activin for the ChIP assays as the induction of Smad2 phosphorylation achieved with Activin in P19 cells is stronger than that achieved with TGF- $\beta$ , probably because they have higher levels of Alk4 than Alk5. P19 cells from a 15cm dish ( $2 \times 10^7$ ) were crosslinked with 1.5% formaldehyde for 10 minutes and the reaction stopped with 0.1875 M glycine. Crude nuclei were prepared, resuspended in 1ml of SDS lysis buffer and sonicated under conditions that reduced the length of the DNA to 200-2000 bp. Chromatin extracts were diluted 10-fold in IP buffer and pre-cleared for 1-2 hours with protein A/G beads coated with 1 mg/ml BSA and 0.4 mg/ml herring sperm DNA (Upstate). Extracts were incubated overnight with 2.5-20  $\mu$ g IgG (Abcam) as appropriate or 10  $\mu$ l anti-PolII (8WG16, Covance) or 10  $\mu$ l anti-Brg1 or 2.5  $\mu$ g anti-trimethyl histone H3 K4 or 2.5  $\mu$ g anti-acetyl histone H3 K18 or 2.5  $\mu$ g anti-acetyl histone H3 K9 or anti-acetyl histone H4 K8 (Abcam) antibodies. Immune complexes were captured for 2 hours with protein A/G beads and washed 3 times each with low salt buffer, high salt buffer and TE. Immune complexes were then eluted and crosslinking reversed overnight. DNA was recovered by phenol extraction and ethanol precipitation. The *lefty1* ARE region and +1 start site were then amplified by quantitative (q) PCR.

### ***Reverse transcription and qPCR***

Total RNA was extracted from P19 cells with Trizol (Invitrogen). RNA (5  $\mu$ g) was reverse transcribed using Superscript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen) with oligo(dT) primers. qPCR was performed with SYBR green PCR master mix (Applied Bioscience) on a Chromo 4 Detector.

For quantitation of mRNA from siRNA experiments PCR reactions were performed in duplicate and the averaged Cts (threshold cycles) were normalized to *GAPDH*. The difference in normalized Cts between the control uninduced TGF- $\beta$  sample (RISC-Free or non-targeting siRNA sample) and the other samples in an experiment allowed the relative expression of a gene to be quantitated with the formula:  $2^{(\text{normalized Cts control}) - (\text{normalized Cts sample})}$ . For quantitation of the ChIP DNA, PCR reactions were performed in triplicate, the averaged Cts were normalized to the lowest IgG and the relative values quantitated with the formula:  $2^{(\text{Avg. Cts IgG} - \text{Avg. Cts sample})}$ . These values were then normalized to the input samples. For primer sequences see below.

### ***Primer sequences for qPCR***

GAPDH Forward,	TGTGATGGGTGTGAACCACGAGAA
GAPDH Reverse,	CTGTGGTCATGAGCCCTTCCACAA
Lefty1 Forward,	CCAACCGCACTGCCCTTAT
Lefty1 Reverse,	CGCGAAACGAACCAACTTGT
Nodal Forward,	TGCCAGACAGAAGCCA ACTGTGTA
Nodal Reverse,	TCGTTTCAGCAGGCTCTGGATGTA
Lefty1 ARE Forward,	AGCGCATTGATAGATACCCCAT
Lefty1 ARE Reverse,	TGGTTTGTATCGCTCAGTGCATAT
Lefty1 +1 Forward,	CCACCCCAGGACCAGCTATAA
Lefty1 +1 Reverse,	GGAAGAGGAGCCTTGGATGTG

### ***Lefty1 promoter***

We have focused on a region of the *lefty1* gene 6 kb upstream of the transcription start site. This region includes an ARE comprising a conserved FoxH1 and Smad binding sites. The ARE maps to within the LPE (lateral plate mesoderm specific enhancer) of the *lefty1* promoter that is described by (Saijoh et al., 1999).

## Supplementary Figure legends

### **Supplementary Figure 1. Purification and recruitment of Smad2P, Smad2, and Smad4 to DNA via the G4-FMSIM.**

(A) Coomassie staining and Western blots of purified Smad proteins using antibodies against unphosphorylated Smad2, phosphorylated Smad2 (Smad2P) or Smad4. (B) Recruitment of Smad2P, unphosphorylated Smad2 and Smad4 by G4-FMSIM was assayed by bandshift using a Gal4 binding site probe. Complexes were confirmed by supershifts with anti-Gal4 and anti-Smad2 antibodies. Whereas addition of Smad4 decreases the mobility and increases the abundance of the complex of Smad2P-G4-FMSIM on the probe, it has little effect on the Smad2-G4-FMSIM complex as expected since it does not form complexes with unphosphorylated Smad2. The Smad2-G4-FMSIM complex does however supershift with both the Gal4 antibody and the Smad2 antibody. Asterisk indicates a non-specific DNA-binding complex.

### **Supplementary Figure 2. Phosphorylated Smad2 recruited to the ARE only activates transcription on a chromatin template.**

(A) Western blots of Flag-XFoxH1b purified from NIH3T3 transfected cells by immunoprecipitation with anti-Flag antibody ( $\alpha$ m2) and elution with Flag peptide. (B) Recruitment of *in vitro* Smad2 complexes by purified Flag-XFoxH1b was assayed by bandshift using an ARE binding site probe. Complexes were confirmed by supershifts with anti-Flag ( $\alpha$ m2) and anti-Smad2 ( $\alpha$ S2) antibodies. The asterisk indicates a non-specific complex. (C) Naked DNA templates of ARE-E4 or G5E4 were used in transcription assays with 2  $\mu$ l (+) or 4  $\mu$ l (++) of Flag-XFoxH1b, 100 ng (+) or 200 ng (++) of Smad2P, Smad4, Gal4 (1-95), G4-AH and G4-p53. (D) Chromatin templates of ARE-E4 were used in transcription assays with Flag-XFoxH1b and recombinant Smad proteins.

### **Supplementary Figure 3. Purification and DNA binding of Gal4 fusion proteins.**

(A) Schematics of Gal4 (1-95), G4-Smad4, G4-Smad2C and G4-Smad4C (top). Coomassie staining of purified Gal4 fusion proteins (bottom). (B) DNA binding ability of the Gal4 fusion proteins was analyzed by bandshift assay using a Gal4 DNA

binding site probe. Complexes were confirmed by supershifts with an anti-Gal4 antibody. (C) Recruitment of Smad2P by G4-Smad4 and G4-Smad4C was assayed by bandshift using a Gal4 DNA binding site probe. Complexes were confirmed by supershifts with the indicated antibodies. In (B) and (C) the asterisk indicates a complex of truncated G4-Smad4 bound to DNA.

**Supplementary Figure 4. Phosphorylated Smad2 complexes do not mediate acetylation of free histones.**

p300 histone acetylation assays performed on free HeLa core histones incubated with 250 ng (+) or 500 ng (++) of Gal4 (1-95), G4-Smad4 and Smad2P. Histone acetylation was assayed by Western blotting with anti-acetyl histone H3 and anti-acetyl histone H4. The levels of Smad2P and G4-Smad4 confirmed with anti-Smad2, and anti-Smad4 antibodies

**Supplementary Figure 5. Phosphorylated Smad2 recruits p300 to mediate acetylation of nucleosomal histone H3 at K9.**

(A) p300 histone acetylation assays on G5E4 dinucleosome template made with recombinant histone octamers incubated with G4-p53 (500 ng), G4-Smad4 alone or with Smad2P (100, 250 or 500 ng). The Western blot was analyzed with an anti-acetyl histone H3 K9 antibody. (B and C) qPCR of the *lefty1* ARE region (B) or +1 transcription start site (C) of chromatin immunoprecipitations with IgG, and anti-acetyl histone H3 K9. ChIPs were performed on extracts from P19 cells treated with 10  $\mu$ M SB-431542 overnight to abolish autocrine signaling and were either uninduced or induced with Activin for 1 hour. The data correspond to the average of triplicate PCRs normalized to IgG and inputs from a representative experiment. The IgG values were set at 1 in the graphs.

**Supplementary Figure 6. Role of Brg1 and Smad2 in the expression of *lefty1* and *nodal* in P19 cells.**

(A) Proteins from P19 cells transfected with siRNA pools against Smad2 or non-targeting (NT), were Western blotted with anti-Smad2 or anti-Smad4 antibodies. (B and C) Expression of *lefty1* (B) or *nodal* (C) was analyzed by reverse transcription and qPCR of mRNA from P19 cells transfected with an siRNA pool against Smad2 or

a non-targeting siRNA. The data correspond to normalized PCR reactions from a representative experiment. Following transfection, samples of cells were treated overnight with SB-431542 to abolish autocrine signaling, washed and then treated -/+ TGF- $\beta$  for 2 hours. All PCRs were performed in duplicate and quantitated relative to *GAPDH*.

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