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Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription

Sarah Ross[1,4], Edwin Cheung[2,3,4], Thodoris G Petrakis[1], Michael Howell[1], W Lee Kraus[2] and Caroline S Hill[1,*]

[1]Laboratory of Developmental Signalling, Cancer Research UK London Research Institute, London, UK, [2]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA and [3]Genome Institute of Singapore, Genome, Singapore

Smads are intracellular transducers for TGF-β superfamily ligands, but little is known about the mechanism by which complexes of receptor-phosphorylated Smad2 and Smad4 regulate transcription. Using an *in vitro* transcription system, we have discovered that, unlike most transcription factors that are sufficient to recruit the basal transcription machinery and therefore activate transcription on both naked DNA and chromatin templates, the Smads only activate transcription from chromatin templates. We demonstrate that Smad2-mediated transcription requires the histone acetyltransferase, p300. Smad2-recruited p300 exhibits an altered substrate specificity, specifically acetylating nucleosomal histone H3 at lysines 9 and 18, and these modifications are also detected on an endogenous Smad2-dependent promoter in a ligand-induced manner. Furthermore, we show that endogenous Smad2 interacts with the SWI/SNF ATPase, Brg1, in a TGF-β-dependent manner, and demonstrate that Brg1 is recruited to Smad2-dependent promoters and is specifically required for TGF-β-induced expression of endogenous Smad2 target genes. Our data indicate that the Smads define a new class of transcription factors that absolutely require chromatin to assemble the basal transcription machinery and activate transcription.

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Introduction

Smads are signal transducers that mediate the cellular responses of the TGF-β family of ligands. Smad2 and Smad3 are receptor-regulated Smads (R-Smads) that are phosphorylated at their C-terminus by activated type 1 receptors in response to TGF-β, Activin or Nodal family members. Upon phosphorylation, Smad2 and Smad3 form homomeric complexes and heteromeric complexes with Smad4 that accumulate in the nucleus where they regulate gene expression.

Smads have two conserved domains, an N-terminal MH1 domain and a C-terminal MH2 domain, separated by a variable linker. The MH2 domain mediates Smad–Smad interactions and interactions between Smads and other proteins and is the site of receptor-mediated phosphorylation in Smad2 and Smad3 (ten Dijke and Hill, 2004; Massagué *et al*, 2005). The Smad3 and Smad4 MH1 domains have weak intrinsic DNA-binding activity, while the Smad2 MH1 domain has no DNA-binding activity. High affinity and specific recruitment of Smads to DNA is achieved by interaction with other DNA-binding factors. For example, in *Xenopus*, activated Smad2–Smad4 complexes are recruited to the Activin-responsive element (ARE) of the *Mix.2* promoter by the forkhead transcription factors XFoxH1a and XFoxH1b (Howell *et al*, 2002) and to the distal enhancer of the *Xenopus goosecoid* promoter by members of the Mix family (Randall *et al*, 2002). Interaction of the FoxH1 and Mix transcription factors with Smad2 is mediated by a conserved proline-rich Smad-interacting motif (SIM) (Randall *et al*, 2002). The FoxH1s additionally contain a Fast/FoxH1 motif (FM), which interacts specifically with phosphorylated Smad2 complexes (Randall *et al*, 2004).

Smad2 and Smad3 synergize with Smad4 to activate gene expression in response to TGF-β ligands (Massagué *et al*, 2005). Fusions of the Smads to Gal4 or LexA DNA-binding domains have suggested that the C-terminal MH2 regions of Smad2, Smad3 and Smad4 have intrinsic transcriptional activity (Liu *et al*, 1996, 1997; Wu *et al*, 1997). In addition, both Smad4 and Smad3 also contain regions within their linkers that interact with the coactivator p300, which are important for transcriptional activation (de Caestecker *et al*, 2000; Wang *et al*, 2005). A number of other coactivators have also been implicated in Smad-regulated transcription. Some of these, like p300, have histone acetyltransferase (HAT) activity, including PCAF and GCN5 and others appear to potentiate TGF-β-induced Smad transcription in a p300/CBP-dependent manner, such as MSG1 (Massagué *et al*, 2005). ARC105/MED15, a component of the mediator complex (Conaway *et al*, 2005), has been shown to interact with Smad2 and Smad3 and to enhance transcription in response to TGF-β ligands (Kato *et al*, 2002). Cofactors that repress Smad-mediated transcription have been identified (for a review, see Massagué *et al*, 2005). TGIF, Ski/SnoN and Evi-1 recruit histone deacetylases to the Smad complexes, while others, such as SNIP, interfere with p300 activity.

Although it is clear that Smads can both activate and repress transcription, and some cofactors are known, the mechanism is not well understood. For example, does

*Corresponding author. Laboratory of Developmental Signalling, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK. Tel.: +44 20 7269 2941; Fax: +44 20 7269 3093; E-mail: caroline.hill@cancer.org.uk
[4]These authors contributed equally to this work

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p300/CBP enhance transcription through acetylation of histones, Smads, or other transcription factors, or by creating a 'bridge' to the basal transcription machinery? Elucidating the mechanism of Smad-mediated transcription *in vivo* has been extremely difficult. Use of mutant Smads is complicated by the fact that they will form complexes with endogenous Smads upon TGF-β stimulation, and it has been difficult to distinguish the Smad sequences required for transcriptional activation *per se* from those required for phosphorylation, complex formation, nuclear accumulation and DNA binding.

Here, we have determined how phosphorylated Smad2-containing complexes activate transcription by developing an *in vitro* transcription system using recombinant Smad proteins. We find that, unlike most well-characterized transcription factors, phosphorylated Smad2-containing complexes only activate transcription from chromatin templates, and not at all from naked DNA. Consistent with this, we find that phosphorylated Smad2-containing complexes recruit p300 to chromatin and specifically acetylate nucleosomal histone H3 at lysines 9 and 18. Ligand-dependent acetylation of these histones is also observed *in vivo* on a Smad2-dependent promoter. Moreover, we demonstrate that Smad2 interacts with Brg1, a component of the SWI/SNF chromatin remodeling complex, in a TGF-β-dependent manner and we show that Brg1 is recruited to target gene promoters and is required for transcription of endogenous Smad2-dependent genes. Our data indicate that the activated Smad2-containing complexes do not activate transcription by directly recruiting basal transcription machinery to the promoter DNA. Rather, they suggest that the Smads recruit the basal transcription machinery indirectly as a result of their ability to orchestrate specific histone modifications and chromatin remodeling.

Results

An in vitro system to study Smad-regulated transcription

To understand how Smads regulate transcription, an *in vitro* transcription assay was developed. A fusion protein comprising the C-terminal region of XFoxH1b, including the FM and SIM Smad2-interaction motifs (Randall *et al*, 2004), fused to the Gal4 DNA-binding domain (G4-FMSIM; Figure 1A) was used to recruit homomeric phosphorylated Smad2 complexes or phosphorylated Smad2–Smad4 complexes to DNA. We

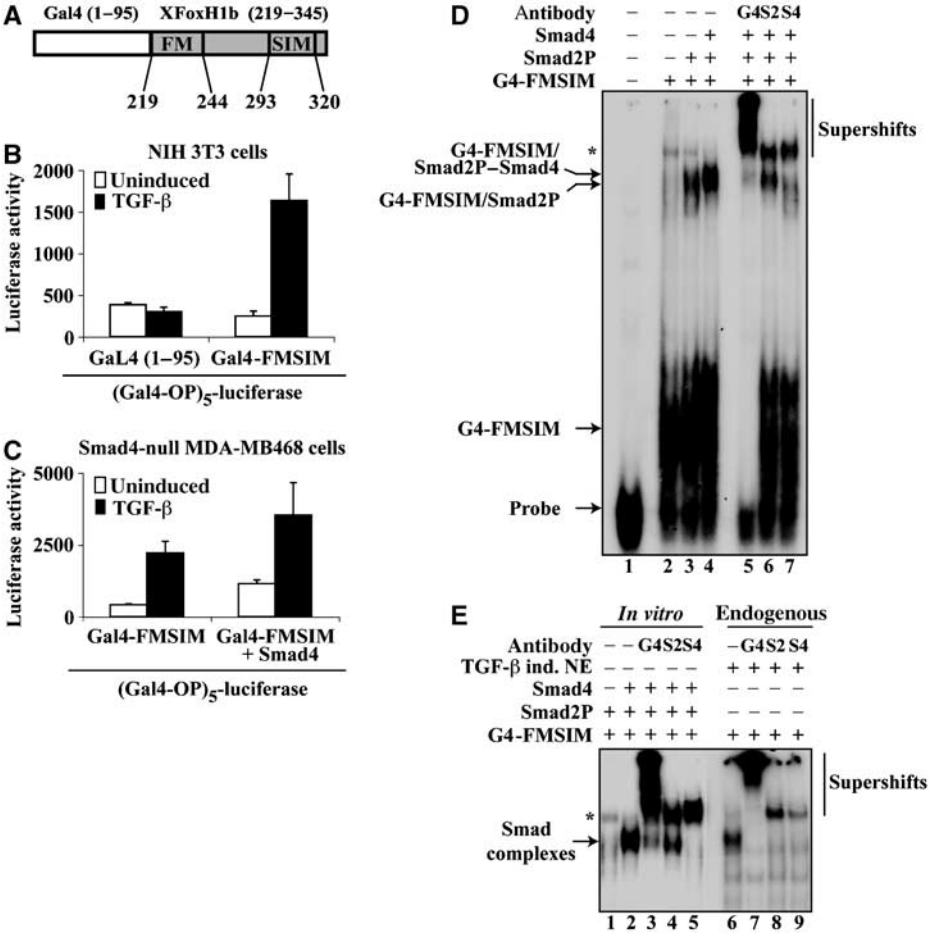

Figure 1 An *in vitro* system for studying Smad2-dependent transcriptional activation. (**A**) A schematic of G4-FMSIM. (**B**) Luciferase reporter assay in NIH 3T3 cells transfected with $(Gal4\text{-}OP)_5$-luciferase and plasmids expressing Gal4 (1–95) or G4-FMSIM. (**C**) Luciferase reporter assays in MDA-MB468 cells transfected with $(Gal4\text{-}OP)_5$-luciferase and plasmids expressing G4-FMSIM alone or with HA-Smad4. (**D**, **E**) Recruitment of *in vitro* or endogenous Smad complexes by recombinant G4-FMSIM was assayed by bandshifts using a Gal4 binding site probe. Complexes were confirmed by supershifts with anti-Gal4 (G4), anti-Smad2 (S2) and anti-Smad4 (S4) antibodies. For *in vitro* Smad complexes, purified proteins were used; for endogenous complexes, nuclear extract from TGF-β-induced HaCaT cells (TGF-β ind.NE) was used. Asterisk indicates a nonspecific DNA-binding complex.

confirmed that G4-FMSIM recruited phosphorylated Smad2-containing complexes to DNA *in vivo* since it specifically conferred TGF-β-induced transcription onto a $(Gal4\text{-}OP)_5$-luciferase reporter in NIH 3T3 cells (Figure 1B). This TGF-β induction was largely Smad4-independent, although it could be potentiated by Smad4 overexpression (Figure 1C).

Recombinant phosphorylated Smad2 (hereafter Smad2P), Smad4 and G4-FMSIM were purified (Supplementary Figure 1A), and bandshift assays used to demonstrate that G4-FMSIM efficiently recruited to a Gal4 binding site, either a recombinant homomeric Smad2P complex (Figure 1D, lanes 2 and 3 and Supplementary Figure 1B) or a Smad2P–Smad4 complex, which could be supershifted by anti-Gal4, anti-Smad2 and anti-Smad4 antibodies (Figure 1D, lanes 4–7). Importantly, when recruited to DNA by recombinant G4-FMSIM, the recombinant *in vitro*-generated Smad2P–Smad4 complex had the same mobility in bandshifts and supershifts as endogenous activated Smad complexes isolated from TGF-β-induced HaCaT nuclear extract (Figure 1E). Hence, the recombinant Smad2P–Smad4 complexes are likely to be correctly folded, and of correct stoichiometry.

Phosphorylated Smad2-containing complexes only activate transcription from a chromatin template

To investigate the transcriptional activity of Smad2P-containing complexes, recombinant Smad2P and Smad4 were recruited by G4-FMSIM to naked DNA templates containing five Gal4 binding sites upstream of the adenovirus E4 (AdE4) promoter (G5E4) (Lin *et al*, 1988; Figure 2A). *In vitro* transcription assays were performed in the presence of HeLa nuclear extract to provide basal transcription machinery and cofactors, and transcriptional activity was measured by the level of E4 transcript assayed by primer extension. Two major products are detected (~45 and 51 nucleotides), as a result of stuttered initiation sites 6 bp apart (Lee and Green, 1987). Surprisingly, recruitment of homomeric Smad2P or heteromeric Smad2P–Smad4 complexes to naked DNA templates did not activate transcription above basal levels, although, the control, Gal4-p53 (G4-p53) efficiently activated transcription on the template containing the Gal4 binding sites (Figure 2A). Addition of nuclear extract from TGF-β-induced HaCaT cells to the transcription assays was unable to stimulate Smad2P-dependent transcription, suggesting that the absence of a TGF-β-dependent cofactor was not the reason for the lack of transcriptional activity of the Smad complexes (data not shown). In addition, titrating amounts of HeLa nuclear extract or levels of activators also failed to reveal any Smad-dependent transcription (data not shown).

In vivo, the assembly of genes into chromatin is vital for regulated gene expression. Even in transient transfections, plasmid DNA templates assemble into nucleosomes (Cereghini and Yaniv, 1984). To determine whether TGF-β-dependent transcription via Smad2P-containing complexes was mediated through the regulation of chromatin structure, transcription assays were performed on the G5E4 template that had been assembled into chromatin (Kraus and Kadonaga, 1998). Recruitment of Smad2P to chromatin templates stimulated transcription 14-fold (Figure 2B). In parallel assays where histones were competed away from the G5E4 templates by competitor DNA, no Smad2P-dependent transcription was seen (data not shown). Smad4 was not essential for Smad2P-dependent transcription on chromatin, although addition of purified Smad4 to a transcription reaction with Smad2P potentiated activation (Figure 2B), consistent with Smad2P–Smad4 complexes being more stable than homomeric Smad2P complexes (Figure 1D; Chacko *et al*, 2004). Transcriptional activation was completely dependent on the recruitment of the Smad2P complexes to DNA by G4-FMSIM (Figure 2C). The same chromatin-dependent transcription by Smad2P complexes was observed when *in vitro* transcription assays were performed on templates containing three AREs regulating the AdE4 gene, and the Smads were recruited by purified XFoxH1b (Supplementary Figure 2).

Therefore, unlike most transcription factors, Smad2P-containing complexes only activate transcription from chromatin templates and have no activity on naked DNA.

Phosphorylation of full-length Smad2 is required for transcriptional activation in vitro

C-terminal phosphorylation of Smad2 is required for Smad complex formation and the nuclear accumulation of Smad2, but a direct role in transcription is unproven. Recombinant unphosphorylated Smad2 (Supplementary Figure 1A) was shown to form a complex with G4-FMSIM on the Gal4 probe (Supplementary Figure 1B), presumably mediated by the XFoxH1b SIM domain, which can bind monomeric Smad2 as well as complexed Smad2 (Randall *et al*, 2002, 2004). However, unlike Smad2P-containing complexes, unphosphorylated Smad2 had no transcriptional activity on a chromatin template alone or with Smad4 and in fact slightly repressed the basal level of transcription (Figure 2D), suggesting that it was recruited to DNA, but interfered with basal transcription.

We examined the role of C-terminal phosphorylation of Smad2 further by fusing domains of unphosphorylated Smad2 to the Gal4 DNA-binding domain (Supplementary Figure 3A). A Gal4 fusion of the Smad2 C-terminal region including most of the linker and MH2 domain (G4-Smad2C) bound DNA efficiently (Supplementary Figure 3B) and was able to activate transcription >4-fold relative to Gal4 (1–95) in chromatin transcription assays (Figure 2E), but had no activity on naked DNA templates (Figure 2A). Therefore, although phosphorylation of the C-terminal region of full-length Smad2 is required to activate transcription on chromatin templates, the C-terminal region of Smad2 by itself has some transcriptional activity on chromatin templates when unphosphorylated.

Smad4 alone has transcriptional activity in vitro, but this is potentiated by Smad2P in Smad2P–Smad4 complexes

To address whether Smad4 alone is transcriptionally active, we investigated the activity of a recombinant fusion of full-length Smad4 to the Gal4 DNA-binding domain (Supplementary Figure 3A). Recombinant G4-Smad4 bound to a Gal4 probe in bandshift assays (Supplementary Figure 3B) and activated transcription on a chromatin template >10-fold relative to Gal4 (1–95) (Figure 2E and F), although not at all on a nonchromatin template (data not shown). G4-Smad4 recruited Smad2P to DNA as shown by bandshift assays (Supplementary Figure 3C) and this recruitment enhanced transcriptional activity on chromatin templates a further six-fold (Figure 2F). Surprisingly, a fusion of the

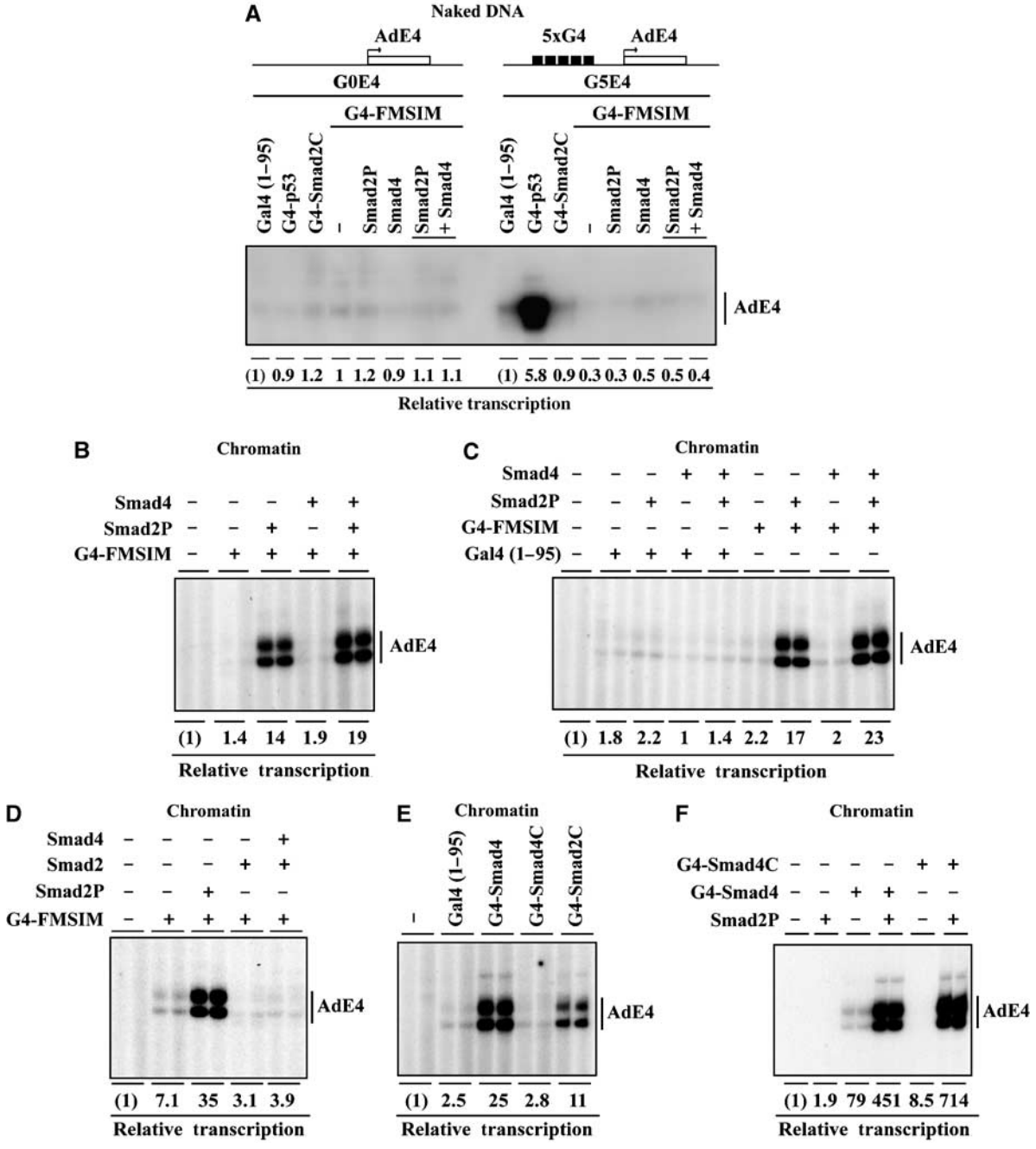

Figure 2 Phosphorylated Smad2-containing complexes activate transcription on chromatin templates, but not on naked DNA. (**A**) A schematic of the plasmid templates used in the *in vitro* transcription assays (top). Naked DNA templates were used with Gal4(1–95), G4-p53, G4-Smad2C and G4-FMSIM with recombinant Smad proteins (bottom). (**B**–**D**) Transcription assays on chromatin templates with G4-FMSIM or Gal4 (1–95) and recombinant Smads as indicated. (**E**) G4-Smad4, G4-Smad4C and G4-Smad2C were analyzed for transcription activity on chromatin templates. (**F**) Smad2P was recruited to the chromatin template by G4-Smad4 or G4-Smad4C and transcriptional activity analyzed. Transcription assays were performed in duplicate and the level of activated transcription was quantitated relative to basal levels of transcription. Between experiments, a certain expected variability in transcriptional induction for G4-FMSIM alone that ranges from two to seven-fold is observed.

Smad4 C-terminal region containing half the linker and the MH2 domain to Gal4 (G4-Smad4C; Supplementary Figure 3A), which was efficiently recruited to DNA (Supplementary Figure 3B and C), was unable to activate transcription on either chromatin or nonchromatin templates (Figure 2E, F and data not shown), suggesting that the Smad4 MH1 domain and/or the N-terminal region of linker is required for transcriptional activation. However, when Smad2P was recruited to DNA by G4-Smad4C (Supplementary Figure 3C), this complex was able to activate transcription on a chromatin template to a very high level (Figure 2F).

Therefore, Smad4 alone is transcriptionally active on chromatin templates *in vitro* and this activity requires more than

the C-terminal region of the protein. Recruitment of Smad2P by either the transcriptionally active full-length Smad4 or the inactive Smad4C substantially increases transcriptional activity to high levels.

The HAT activity of p300 is required for Smad2 to activate transcription

We next investigated the possible identity of proteins recruited to chromatin by Smad4–Smad2P complexes, and examined whether they would be able to modify and remodel chromatin. CBP and p300 are coactivators implicated in Smad-mediated transcription (Massagué *et al*, 2005) that possess intrinsic HAT activity. Addition of a specific inhibitor of p300 HAT activity, LysCoA (Lau *et al*, 2000), reduced Smad2P-mediated transcription on chromatin templates in a dose-dependent manner (Figure 3A), indicating that p300 is required for Smad2P to activate transcription. Conversely, overexpression of p300 enhanced Smad2P-mediated transcription on chromatin (Figure 3B). Moreover, immunoprecipitation assays with recombinant proteins showed that p300 specifically interacted with phosphorylated Smad2P, but not unphosphorylated Smad2 (Figure 3C). Depletion of p300 in NIH 3T3 cells by siRNA (Figure 3D) reduced the TGF-β-induced transcription of a Smad2-dependent reporter (ARE-luciferase with XFoxH1b; Figure 3E and F), indicating that p300 is required *in vivo* for Smad2-dependent transcriptional activation.

To determine whether p300 recruited to chromatin by Smad2P acetylates histones, HAT assays were performed on a 450 bp fragment of G5E4 assembled into a dinucleosome. The p300 protein alone was unable to acetylate the histones within the dinucleosome template, although it could acetylate free core histones efficiently (Figure 4A). However, when Smad2P was recruited to the dinucleosome by G4-Smad4, histones H3 and H4 were acetylated by p300 in a Smad2P dose-dependent manner (Figure 4A). G4-Smad4 alone

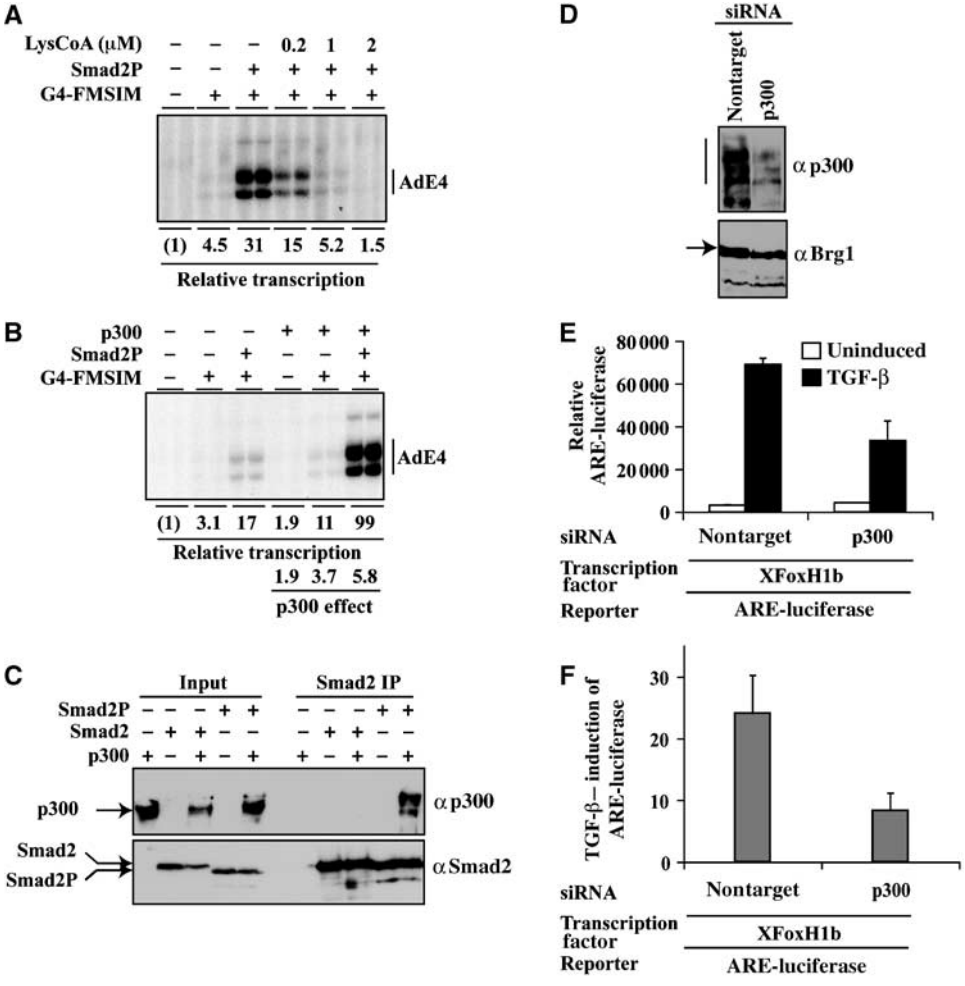

Figure 3 p300 is required for Smad2 complexes to activate transcription. (**A**) Transcription assays on chromatin templates using Smad2P recruited by G4-FMSIM in the presence of the p300 inhibitor, LysCoA. (**B**) p300 synergizes with Smad2P to induce transcription *in vitro* on chromatin templates. Transcription assays were performed in duplicate and the level of transcription quantitated relative to basal levels. (**C**) Interaction of recombinant p300 with recombinant Smad2 and Smad2P was assayed by immunoprecipitation with an anti-Smad2 antibody and Western blot analysis with anti-p300 and anti-Smad2 antibodies. The unphosphorylated Smad2 has a short N-terminal linker, which accounts for its slight decrease in mobility relative to Smad2P. (**D**) Proteins from NIH 3T3 cells transfected with siRNA pools against p300 or a nontarget control were analyzed by Western blotting using anti-p300 or anti-Brg1 antibodies. (**E**) Luciferase reporter assay in NIH 3T3 cells transfected with ARE-luciferase, a plasmid expressing XFoxH1b and siRNA pools against p300 or nontarget control. (**F**) Fold induction of ARE-luciferase in response to TGF-β from four independent siRNA experiments.

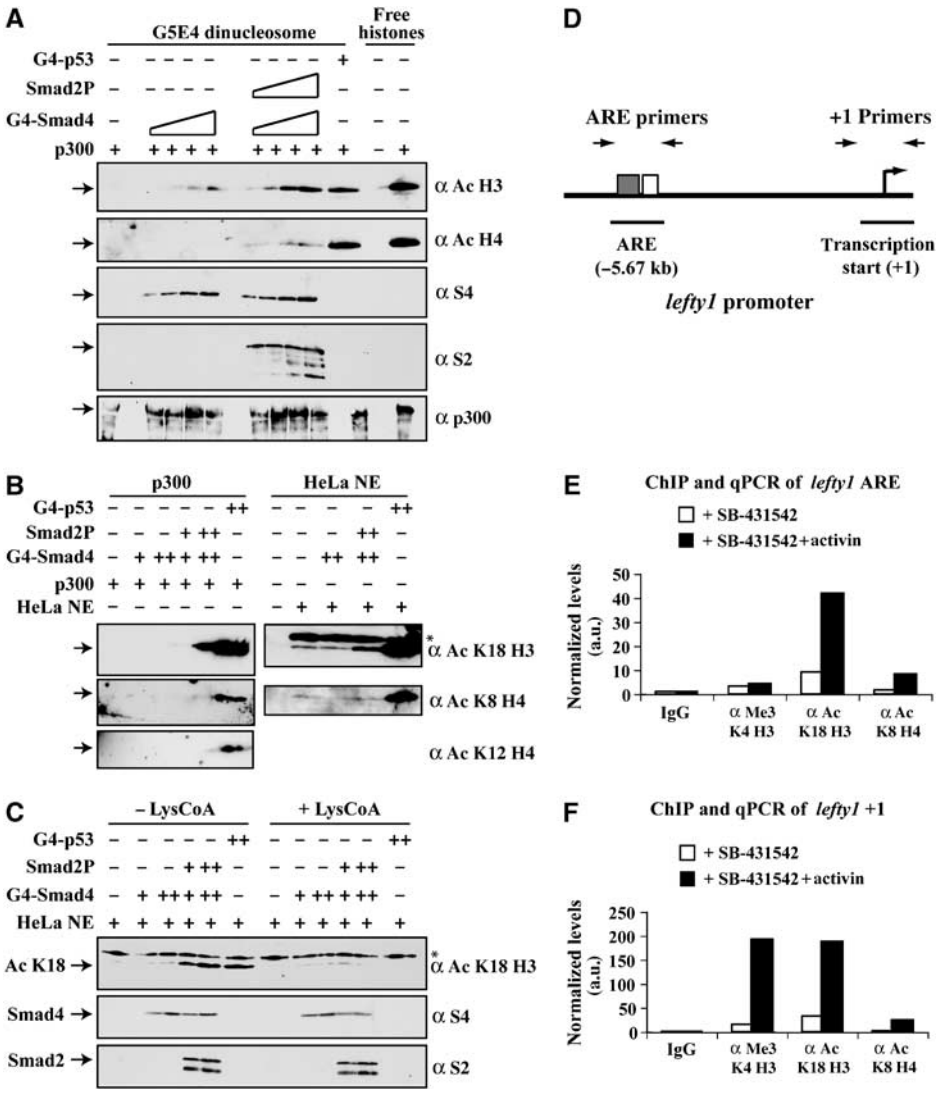

Figure 4 Smad2P-containing complexes recruit p300 to preferentially acetylate nucleosomal histone H3. (**A**) p300 histone acetylation assays performed on G5E4 dinucleosome templates or free core histones incubated with G4-p53 (250 ng) or G4-Smad4 alone or with Smad2P (50, 100, 250, 500 ng each). For (A) histones were prepared from HeLa cells. (**B**) p300 or HeLa nuclear extract-dependent histone acetylation assays on G5E4 dinucleosome templates incubated with 200 ng (+) or 500 ng (++) G4-p53, G4-Smad4 and Smad2P. (**C**) HeLa nuclear extract-dependent histone acetylation assays on G5E4 templates −/+ LysCoA (1 μM final concentration) with 500 ng (+) or 750 ng (++) G4-p53, G4-Smad4 and Smad2P. For (B) and (C) recombinant histone octamers were used. Western blotting was performed with anti-acetyl histone H3, anti-acetyl histone H4, anti-acetyl histone H3 K18, anti-acetyl histone H4 K8 and anti-acetyl histone H4 K12 antibodies as indicated. The levels of Smad2P, G4-Smad4 and p300 were confirmed as indicated. In (B) and (C), the asterisk indicates a non-specific band. (**D**) Schematic of the *lefty1* promoter showing the location of the primers used in the ChIP assays. The ARE, which contains a FoxH1 (gray box) and Smad (white box) binding site is shown, and the start of transcription (+1). (**E, F**) qPCR of the *lefty1* ARE region (E) or +1 transcription start site (F) from ChIP assays using IgG, anti-trimethyl histone H3 K4, anti-acetyl histone H3 K18 and anti-acetyl histone H4 K8 antibodies. ChIPs were performed on extracts from P19 cells treated with 10 μM SB-431542 overnight to abolish autocrine signaling and were either uninduced or induced with activin for 1 h. The data correspond to the average of triplicate PCRs normalized to IgG from a representative experiment. The IgG values were set to 1.

stimulated low levels of p300-mediated acetylation of H3 within the nucleosome (Figure 4A), consistent with the ability of Smad4 to interact with p300 (de Caestecker *et al*, 2000) and its transcriptional activity. Importantly, G4-Smad4 and Smad2P mediated acetylation of nucleosomal histones, but not free histones. In fact, in the presence of G4-Smad4 or Smad2P, the ability of p300 to acetylate free histones is actually reduced (Supplementary Figure 4).

These data indicate that the HAT activity of p300 is required for Smad2P complexes to activate transcription and that Smad4 alone or in combination with Smad2P recruits p300 to chromatin to facilitate acetylation of nucleosomal histones.

Smad2P-recruited p300 preferentially acetylates histone H3

Acetylation of histone H3 was significantly more efficient than acetylation of histone H4 when p300 was recruited by G4-Smad4–Smad2P complexes, whereas p300 recruited by G4-p53 acetylated histones H3 and H4 to the same extent (Figure 4A). These differences may reflect a preference of the p300 recruited by Smad2P complexes to acetylate lysine

residues within the histone H3 tail. To further analyze this possibility, the acetylation of specific lysine residues within the H3 and H4 tails known to be targeted by p300 (Schiltz *et al*, 1999) was studied.

p300 acetylated lysines 18 and 9 of H3 when recruited to the dinucleosome by either G4-Smad4–Smad2P or G4-p53 (Figure 4B; Supplementary Figure 5A). In contrast, lysines 8 and 12 of nucleosomal histone H4 were only acetylated to high levels by G4-p53-recruited p300 (Figure 4B). Indeed, none of the lysine residues within H4 that were tested (K5, K8, K12 or K16) were significantly acetylated when p300 was recruited to nucleosomes by Smad2P complexes (Figure 4B; data not shown). The same histone H3 specificity was observed when HeLa nuclear extract was used as a source of HAT activity in the acetylation assays (Figure 4B), and this acetylation was abolished by the p300 HAT inhibitor LysCoA (Figure 4C). These data indicate that the endogenous HAT activity recruited by Smad2P complexes from HeLa nuclear extract is likely to be p300. In these experiments, no histone acetylation mediated by G4-Smad4 alone was observed, probably because the level of acetylation is too low to be detected in these assays.

To test whether these histone acetylations occurred on the promoter of endogenous Smad2-dependent genes, we used the mouse embryonal carcinoma cell line, P19, which contains endogenous FoxH1 and expresses the genes *lefty* and *nodal* in response to Activin, Nodal or TGF-β via AREs in their promoters (Saijoh *et al*, 2000) in a Smad2-dependent manner (Supplementary Figure 6A–C). We studied histone modification of the *lefty1* promoter, which contains an ARE comprising FoxH1 and Smad binding sites approximately 5.6 kb upstream of the transcription start site (+1) (Figure 4D; Supplementary data). To assay the response of P19s to ligand, cells were treated overnight with an ALK5 inhibitor SB-431542 (Inman *et al*, 2002) to inhibit autocrine TGF-β/Activin/Nodal signaling, the SB-431542 was washed out and the cells were induced with Activin for 1 h. ChIPs using specific acetylated histone antibodies confirmed that the *lefty1* promoter was acetylated at K18 and K9 of histone H3 in response to ligand at both the ARE region and at the transcription start site (Figure 4E and F; Supplementary Figure 5B and C), which is consistent with our *in vitro* results and suggests that p300 is recruited to the *lefty1* promoter by Smad2 complexes *in vivo*. The ARE and transcription start site are also weakly acetylated at K8 of histone H4 (Figure 4E and F), suggesting that another HAT activity is recruited to the *lefty1* promoter *in vivo* by factors other than the Smads. RNA polymerase II recruitment and tri-methylation of histone H3 K4, both hallmarks of active transcription, were only significantly observed at the transcription start site of the *lefty1* promoter as expected (Figure 4E and F; Supplementary Figure 5B and C). The presence of acetylated histones at both the ARE and transcription start site suggests either a looping of the promoter or a spreading of the histone modifications along the promoter in response to ligand.

These results show that the substrate specificity of p300 when recruited to nucleosomes by Smad2P complexes is altered such that it preferentially acetylates nucleosomal histone H3 at lysines 9 and 18. These specific histone modifications are also seen on an endogenous Smad2-dependent promoter, suggesting that p300 is recruited *in vivo* by Smad2 complexes.

Smad2P interacts with Brg1, a component of the SWI/SNF remodeling complex and is required for TGF-β-induced transcriptional activity

Since it is unlikely that acetylation of nucleosomes is sufficient for transcriptional activation in the absence of chromatin remodeling, we investigated whether Smad2P could interact with chromatin remodeling complexes. Of the four known classes of chromatin remodeling complexes, the SWI/SNF chromatin remodeling complexes have been associated with transcriptional regulation of a subset of target genes (Narlikar *et al*, 2002). Therefore, we investigated whether Brg1, the ATPase subunit of the human SWI/SNF complexes BAF and PBAF, could interact with Smad2. Immunoprecipitations with an antibody to endogenous Brg1 co-immunoprecipitated Smad2, Smad3 and Smad4 from TGF-β-induced HaCaT nuclear extracts, but not from uninduced extracts (Figure 5A). In reciprocal experiments, an antibody against endogenous Smad2 and Smad3 co-immunoprecipitated endogenous Brg1 in a TGF-β-dependent manner (Figure 5B). The HaCaT cell lines used for these experiments express different EGFPSmad2 fusions (Figure 5A; Schmierer and Hill, 2005). Brg1 clearly interacts with the C-terminal region of Smad2, but not Smad2 containing the D300H mutation, a Smad2 mutant that is phosphorylated but can no longer form Smad complexes (Figure 5A; Schmierer and Hill, 2005). Thus, the interaction of Brg1 with Smad2 does not require the MH1 domain, but does require Smad complex formation.

To determine whether the interaction between Brg1 and Smad2 was functionally relevant for Smad2-dependent transcription, we depleted nuclear extract of Brg1 and showed that this inhibited Smad2P-dependent transcription *in vitro* (Figure 5C). Furthermore, levels of Brg1 and Brm, an alternative SWI/SNF ATPase subunit, were knocked down by siRNA in NIH 3T3 cells (Figure 5D). Reporter assays using a Smad2-dependent promoter (ARE-luciferase with XFoxH1b) showed that knockdown of Brg1, but not Brm, or expression of a nontargeting siRNA, impaired TGF-β-induced transcriptional activity (Figure 5E and F). Therefore, SWI/SNF chromatin remodeling complexes containing the Brg1 ATPase, but not Brm, are required for TGF-β transcriptional activation by Smad2.

Brg1 is required for TGF-β induction of endogenous Smad2-dependent genes

To examine the role of Brg1 on the regulation of endogenous Smad2-dependent genes, Brg1 protein levels were knocked down by siRNA in P19 cells (Figure 6A) and the TGF-β induction of the *lefty1* and *nodal* genes examined. Knockdown of Brg1 significantly impaired the acute TGF-β induction of *lefty1*, compared to an siRNA control (Figure 6B and D). This was specific, as knockdown of Brg1 had no effect on *GAPDH* levels. Interestingly, expression of *lefty1* in response to autocrine TGF-β signals was less affected by Brg1 knockdown than the acute response (Figure 6D), suggesting that Brg1 is required for induction of transcription in response to TGF-β, but not its maintenance. TGF-β-induced expression of *nodal* was also reduced by siRNAs targeting Brg1 (Figure 6C), but to a lesser extent than *lefty1*, which might reflect the importance of other signaling pathways in the regulation of the *nodal* gene. Indeed, treatment of P19 cells with SB-431542 is unable to completely inhibit

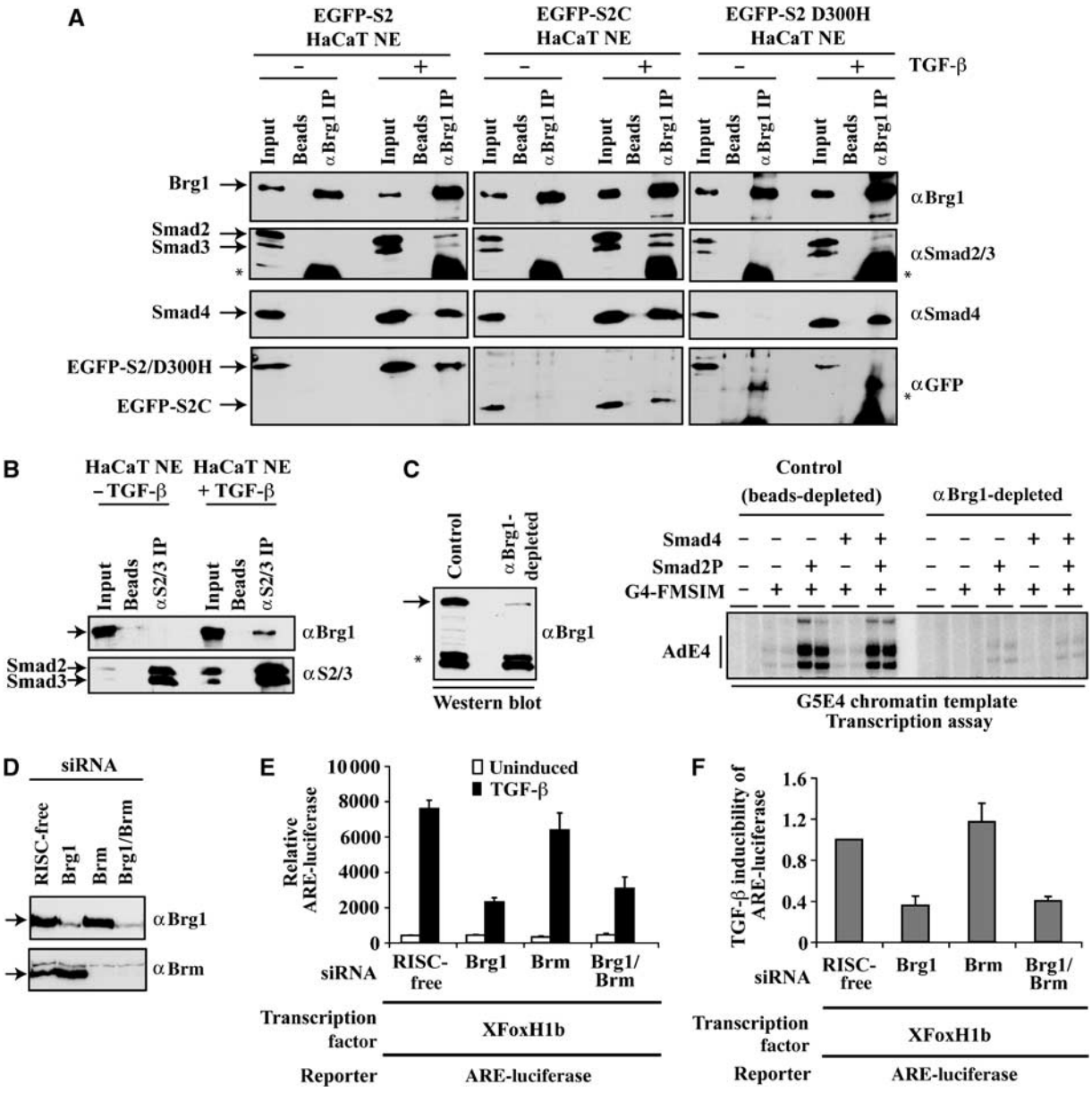

Figure 5 The SWI/SNF component, Brg1, interacts with Smad complexes and is required for TGF-β-activated transcription. (**A, B**) Proteins were immunoprecipitated with immobilized anti-Brg1 (A) or immobilized anti-Smad2/3 (αS2/3) (B) or protein G beads alone (A, B) from HaCaT nuclear extracts (HaCaT NE) that were either uninduced or induced for 1 h with TGF-β. In (A), the HaCaTs were stably expressing EGFP-tagged full-length Smad2 (EGFP-S2), Smad2 linker + MH2 (EGFP-S2C) or Smad2 (D300H) (EGFP-S2 D300H). Immunoprecipitated proteins, together with input protein samples, were analyzed by Western blotting using anti-Brg1, anti-Smad2/3, anti-Smad4 and anti-GFP antibodies as indicated. The asterisk indicates an IgG band. (**C**) Depletion of Brg1 from HeLa nuclear extract (left panel) inhibited Smad2P-dependent transcription *in vitro* (right panel). The asterisk indicates a background band. (**D, E**) NIH 3T3 cells were transfected with siRNA pools against Brg1, Brm or an RISC-free control and analyzed by Western blotting using anti-Brg1 or anti-Brm antibodies (D), or were further transfected with ARE-luciferase, a plasmid expressing XFoxH1b and analyzed for luciferase activity (E). (**F**) A graph representing the fold induction of ARE-luciferase in response to TGF-β from two independent siRNA experiments.

expression of *nodal* (Figure 6C and data not shown), suggesting that signals other than TGF-β also regulate its expression. The levels of Brg1 on the *lefty1* promoter are increased following ligand induction (Figure 6E and F), consistent with the role of Brg1 in Smad2-mediated transcription.

These data show that Brg1 is required for the efficient TGF-β induction of Smad2-dependent target genes *in vivo* and that Brg1 is increased at the *lefty1* promoter following ligand induction. This indicates that an SWI/SNF chromatin remodeling complex containing Brg1 is recruited to the promoters of TGF-β-dependent target genes by activated Smad2-containing complexes to remodel chromatin and induce gene expression.

Discussion

Phosphorylated Smad2-containing complexes only activate transcription on chromatin templates

To study how the Smads activate transcription once bound to DNA, we developed an *in vitro* transcription assay using

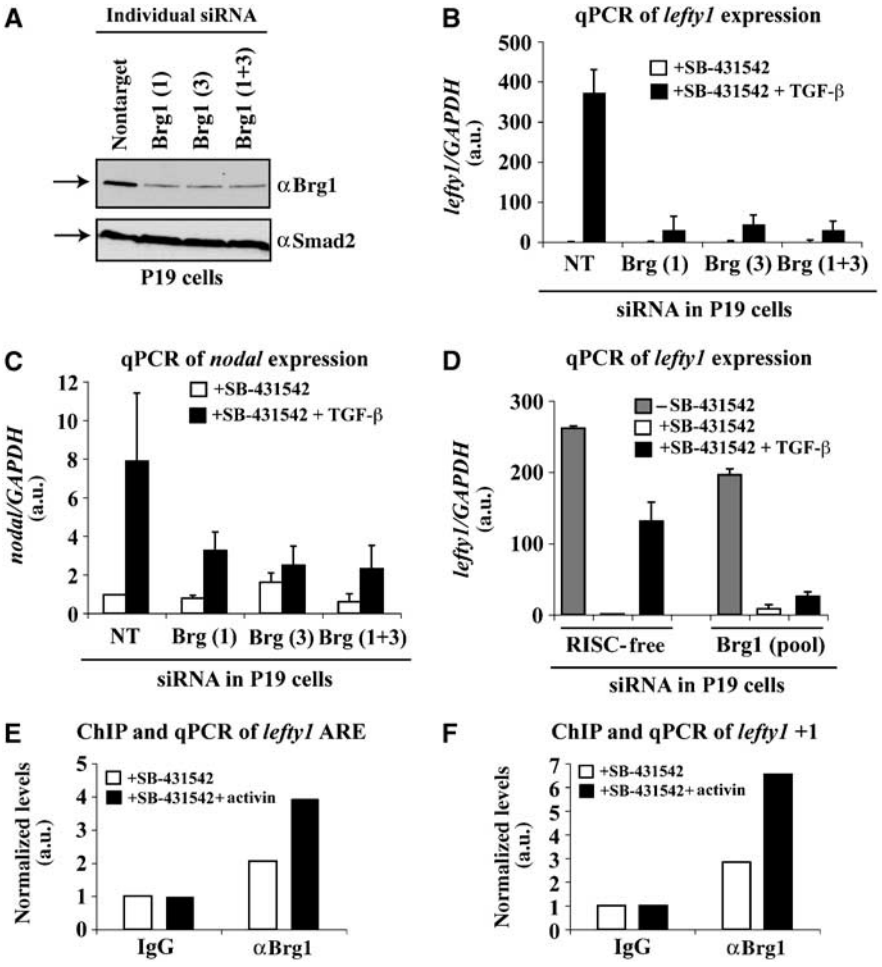

Figure 6 Brg1 is required for the TGF-β-induced expression of *lefty1* and *nodal in vivo* and binds to the *lefty1 promoter*. (**A**) Proteins from P19 cells transfected with individual siRNA duplexes against Brg1 (1 or 3) or a nontargeting siRNA were analyzed by Western blotting with anti-Brg1 or anti-Smad2 antibodies. (**B–D**) Levels of *lefty1* (B, D) or *nodal* (C) mRNA were measured by reverse transcription and qPCR of RNA isolated from P19 cells transfected with individual (Brg1 or Brg3) or a pool of siRNA duplexes targeting Brg1 and either nontargeting (NT) or RISC-free siRNA as controls. Following transfection, samples of cells were treated overnight with SB-431542 to abolish autocrine signaling, washed and then treated −/+ TGF-β for 2 h. The data for (B) and (C) represent the average of four PCR reactions from a representative experiment. The data for (D) correspond to duplicate PCR reactions from a representative experiment. All PCRs were performed in duplicate and quantitated relative to *GAPDH*. (**E, F**). qPCR of the *lefty1* ARE region (E) or +1 transcription start site (F) from ChIPs with IgG or anti-Brg1 antibody. ChIPs were performed on extracts from P19 cells treated with SB-431542 overnight to abolish autocrine signaling and then treated −/+ activin for 1 h. The data correspond to the average of triplicate PCRs normalized to IgG from a representative experiment. The IgG values were set at 1.

recombinant Smad2P and Smad4 proteins. Surprisingly, Smad2P–Smad4 complexes were unable to activate transcription on naked DNA templates. However, the same complexes efficiently activated transcription on chromatin templates. The absolute requirement by the Smads for a chromatin template for transcriptional activation was unexpected, as most other transcription factors tested in this type of assay, for example, SRF, Sp1, p53, NF1, estrogen receptor isoforms and vitamin D receptor, are able to activate transcription on naked DNA templates at least to some extent (Jones *et al*, 1987; Norman *et al*, 1988; Ryu *et al*, 1999; Lemon *et al*, 2001; Cheung *et al*, 2003).

The restriction of Smad2P-mediated transcription *in vitro* to chromatin templates sheds important light on the mechanism of transcriptional regulation by the Smads. That most transcription factors activate transcription on naked DNA reflects their ability to assemble directly the RNA polymerase II preinitiation complex on promoter DNA. Our data indicate that active Smad2P-containing complexes cannot do this, despite the known interaction between Smad2 and the mediator component, ARC105/Med15 (Kato *et al*, 2002). Instead, Smad2P-containing complexes strictly require a chromatin template to mediate transcription, suggesting that the chromatin template is directly involved in the transcriptional activation mechanism. We propose that the histone modifications and remodeling of the chromatin generated by p300, Brg1 and probably additional enzymes recruited by Smad2P complexes, act with Smad2P to promote directly recruitment of the general transcription machinery to DNA to activate transcription (Figure 7). For example, histone H3 acetylation induced by Smad2P-bound p300 could aid recruitment of the TFIID complex via the known interaction between the bromodomain of $TAF_{II}250$ and acetylated lysine residues (de la Cruz *et al*, 2005). In addition, activation of transcription via Smad2P complexes in chromatin may also involve antirepression of the steric constraints of the chromatin structure

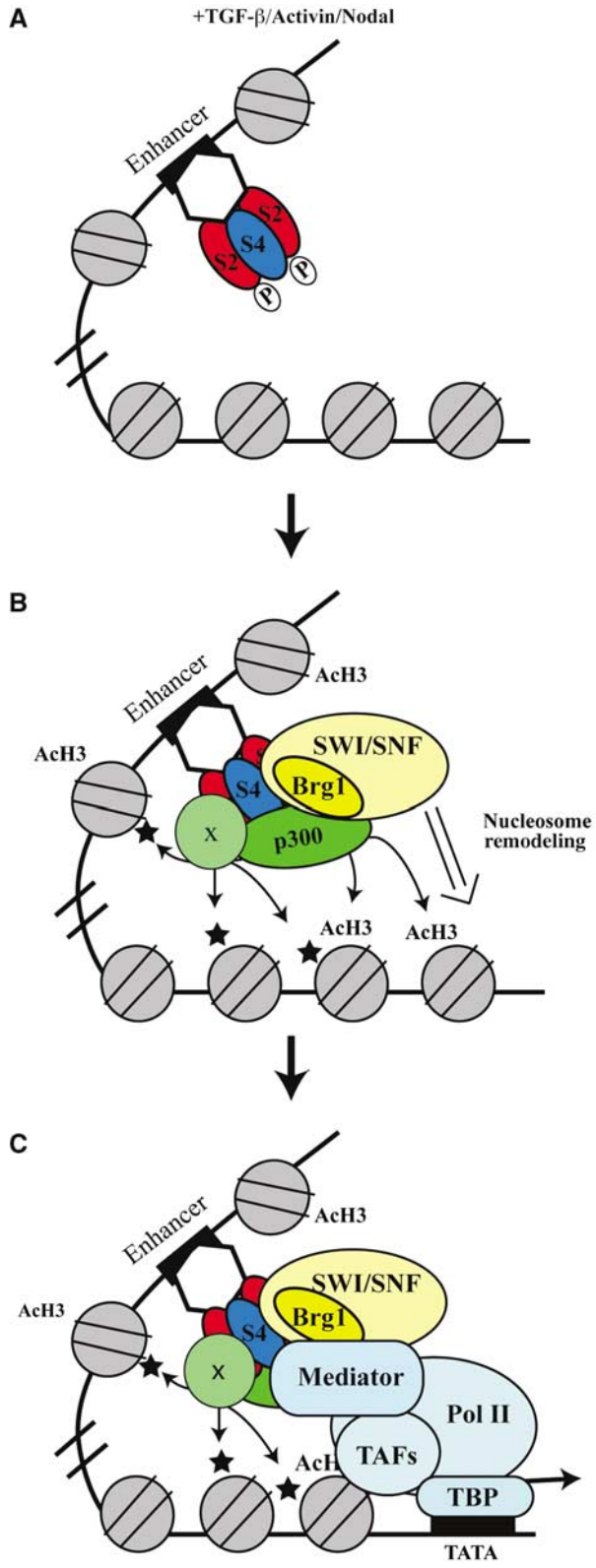

Figure 7 Proposed model of Smad2P-mediated transcription on chromatin. (**A**) In response to TGF-β, Activin or Nodal, Smad2P-containing complexes are recruited to target promoters by transcription factors. (**B**) The Smad2P-containing complexes recruit p300, SWI/SNF and probably other modifiers (indicated by x) to modify (indicated by AcH3 and or a star for unknown modifications) and remodel (indicated by the white arrow) the chromatin. (**C**) The modified and remodeled chromatin together with Smad2P-containing complexes recruits the RNA Pol II transcription machinery to the promoter to activate transcription.

(Roeder, 2005), allowing the RNA Pol II transcription machinery access to the DNA, as has been suggested for *Drosophila* GAGA factor, which plays a role in establishing nucleosome-free regions within the genes that it regulates (Lehmann, 2004).

Requirements for Smads to activate transcription on chromatin

Recruitment of homomeric Smad2P complexes is sufficient to activate transcription both *in vitro* on a chromatin template and *in vivo* on a transfected reporter plasmid. Heteromeric Smad2P–Smad4 complexes only stimulated transcription on chromatin templates marginally better than homomeric complexes. Moreover, recruitment of Smad2P to DNA by G4-Smad4 or G4-Smad4C substantially enhanced transcription on chromatin. Thus, in these assays, Smad4 is not required for efficient Smad2-dependent transcription, and in a heteromeric complex, Smad2P appears to be the major activating component. The essential role for Smad4 in the transcriptional regulation of many TGF-β ligand target genes may therefore more reflect the importance of Smad4 in stabilizing the binding of Smad complexes to DNA (Liu *et al*, 1997), rather than Smad4's transcriptional activity *per se*.

Our *in vitro* system has demonstrated that phosphorylation of full-length Smad2 is required to activate transcription on a chromatin template, but is not essential when the MH1 domain has been deleted. These results are consistent with the C-terminal MH2 domain of Smad2 having inherent transcriptional activity that is inhibited in full-length unphosphorylated Smad2 by the N-terminal MH1 domain (Liu *et al*, 1996; Hata *et al*, 1997). Phosphorylation of the C-terminus of Smad2 unmasks this activity in the context of the full-length protein, while not being directly involved in the transcription process.

Full-length Smad4 alone can activate transcription *in vitro* when recruited to DNA via fusion to the Gal4 DNA-binding domain. As for Smad2P, this transcription was dependent on chromatin, suggesting that Smad4 can also recruit enzymes that modify and remodel chromatin. Indeed, we have shown that G4-Smad4 can recruit p300 to chromatin to acetylate histone H3, although less efficiently than Smad2P. Smad4 fusions can also activate transcription in yeast (Wu *et al*, 1997). In contrast, in mammalian cells, full-length G4-Smad4 alone failed to activate transcription from a reporter plasmid in the absence of TGF-β (Liu *et al*, 1996; Feng *et al*, 1998), perhaps because of the presence *in vivo* of corepressors such as SnoN, which repress Smad4's inherent transcriptional activity (Stroschein *et al*, 1999). Thus, Smad4 contains intrinsic transcriptional activity, which, unlike that of Smad2, is not masked in the full-length protein, but may be regulated *in vivo* in unstimulated cells by repressor proteins that are absent in our *in vitro* assays.

Active Smad complexes facilitate acetylation of histone H3 through recruitment of p300 to chromatin

Acetylation of histones in chromatin is commonly associated with transcriptionally active genes, and p300 and CBP are HATs implicated in TGF-β-induced transcription, although their mechanism has remained obscure (Massagué *et al*, 2005). The coactivator function of p300/CBP has been linked to multiple mechanisms, including histone acetylation, transcription factor acetylation and formation of direct

interactions with the basal transcription machinery (Vo and Goodman, 2001). Our study has demonstrated *in vitro* and *in vivo* that p300's HAT activity is essential for Smad2P to activate transcription and that Smad2P and Smad4 recruit p300 to chromatin to acetylate nucleosomal histones. The level of p300-directed histone acetylation correlated with the level of transcriptional activity of the Smad complexes on chromatin.

Formation of a complex with Smad2P inhibited the ability of p300 to acetylate free histones, while promoting its ability to acetylate nucleosomal histones. Furthermore, while p300 recruited to chromatin by Gal4-p53 acetylated histones H3 and H4 to a similar level, p300 recruited by the Smad2P complexes acetylated H3 significantly better than H4. The ability of Smad2P complexes to alter the specificity of p300 HAT activity to preferentially acetylate nucleosomal histone H3 was confirmed using antibodies against specific lysine residues in H3 and H4. Importantly, we also demonstrate that the endogenous HAT activity recruited by Smad2P complexes from nuclear extract is p300, and that *in vivo* the promoter of a Smad2-dependent target gene is acetylated at histone H3 in a TGF-β ligand-dependent manner. The mechanism by which Smad2P alters the specificity of p300 HAT activity is not yet known, but may result from steric constraints, or from direct effects of Smad2P on the enzymatic activity of p300. We propose that this preferential acetylation of histone H3 is important for Smad2P-mediated transcription on a chromatin template. Indeed, acetylation of lysine 18 on histone H3 has been associated with increased recruitment of other histone-modifying factors such as CARM1 to chromatin (Daujat *et al*, 2002).

Brg1-containing SWI/SNF remodeling complexes are required for Smad2-dependent transcription

In addition to histone-modifying enzymes, ATP-dependent chromatin remodeling complexes are essential for the activation of transcription on chromatin. The multisubunit complexes of the SWI/SNF family are one class of such enzymes involved in transcriptional regulation (Narlikar *et al*, 2002). We have found that endogenous Smad2 associates in a TGF-β-dependent manner with endogenous Brg1, the ATPase component of the mammalian SWI/SNF complexes, BAF and PBAF. While this paper was in revision, another group also reported the interaction *in vitro* between pseudophosphorylated Smad2 linker plus MH2 domain and Brg1 (He *et al*, 2006). Here we show that Brg1, but not Brm, is required for TGF-β activation of a Smad2-dependent reporter and for efficient induction of the TGF-β-responsive genes *lefty1* and *nodal in vivo*. Furthermore, we have shown that Brg1 is recruited to the *lefty1* promoter *in vivo*. We infer that active Smad2-containing complexes recruit Brg1-containing SWI/SNF remodeling complexes to chromatin *in vivo*. Interestingly, reduction of Brg1 levels affects gene expression in response to acute TGF-β induction more than that in response to autocrine signals, suggesting that during autocrine signaling, the promoter chromatin of these genes is already accessible to the transcription machinery and therefore does not require Brg1.

It is intriguing that, like components of the TGF-β signaling pathway (Levy and Hill, 2006), mutations in subunits of the SWI/SNF complex (including Brg1) have been identified in some cancers and these subunits act as tumor suppressors (Roberts and Orkin, 2004). The new connection between Brg1 and the TGF-β pathway that we uncover raises the intriguing possibility that the tumor suppressor activity of the SWI/SNF complexes may be mediated through the TGF-β pathway.

In conclusion, we have shown that Smad2P-containing complexes activate transcription through chromatin remodeling. This chromatin remodeling is achieved, at least in part, through the recruitment of the HAT p300 and chromatin remodelers of the SWI/SNF family. It is likely that other chromatin-modifying complexes are also involved in Smad2-mediated transcription and their identification and role will be of great interest in the future.

Materials and methods

Plasmids, recombinant proteins, bandshift assays and immunoprecipitations

For details of plasmids, recombinant proteins, bandshift assays and immunoprecipitations, see Supplementary data.

Cell culture, transfections and reporter assays

HaCaT, NIH 3T3 and MDA-MB468 cells were maintained, and plasmid transfections and reporter assays were performed as described (Pierreux *et al*, 2000). P19 cells were grown as described previously (Rudnicki and McBurney, 1987). The EGFPSmad2 HaCaT cell lines are described in Schmierer and Hill (2005). siRNA pools (a mix of four different siRNA duplexes) and individual siRNA duplexes were obtained from Dharmacon (see Supplementary data) and transfected at a final concentration of 75 nM into P19 or NIH 3T3 cells using Dharmafect 3 reagent (Dharmacon). Following an overnight incubation, cells were plated for reporter assays, RNA or protein preparation. P19 cells were treated overnight with 10 μM SB-431542 (Inman *et al*, 2002) to abolish autocrine signaling, before washing and inducing with 2 ng/ml TGF-β for 2 h.

Chromatin immunoprecipitations (ChIPs)

ChIP experiments were performed as described (Alberts *et al*, 1998), with minor modifications (see Supplementary data).

In vitro transcription assays

Transcription assays on naked DNA templates were performed as described previously (Lee and Green, 1987), using HeLa nuclear extract (Cil Biotech). Immunodepletion of Brg1 was performed overnight using anti-Brg1 (Upstate) or beads alone as a control. A typical transcription reaction contained 200 ng of template DNA (G0E4 or G5E4), 100 ng of Gal4 fusion protein, 50 ng of Smad2P and 200 ng of Smad4. DNA templates (G5E4) were assembled into chromatin or mock assembled for transcription assays using *Drosophila* S190 extract and *in vitro* transcription reactions were performed on them as described (Kraus and Kadonaga, 1998). A typical reaction contained 50 ng of Gal4 fusion protein, 50 ng of Smad2P and 100 ng of Smad4.

Core histone preparation and assembly of dinucleosomes and nucleosome arrays and histone acetylation assays

Core histones were prepared from HeLa cells (Cote *et al*, 1995). Recombinant histone octamers were prepared as described (Luger *et al*, 1997). DNA was assembled into a dinucleosome by serial salt dilutions as described (Steger *et al*, 1998). Histone acetylation assays were performed as described in the Supplementary data.

Reverse transcription and quantitative PCR (qPCR)

Reverse transcription and qPCR was performed as described in the Supplementary data.

Supplementary data

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

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