An Essential Role for Mad Homology Domain 1 in the Association of Smad3 with Histone Deacetylase Activity*†

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The Smads are a family of sequence-specific DNA-binding proteins that modulate transcription in response to transforming growth factor β (TGFβ) by recruiting transcriptional activators like the histone acetyltransferase, p300/CREB, or repressors like the histone deacetylase, HDAC1, to TGFβ target genes. The association of Smads and HDAC1 is mediated in part by direct binding of Smads to the HDAC1-associated proteins, TG-interacting factor, c-ski, and SnoN. Although ectopic expression of these proteins inhibits Smad-activated transcription, the contribution of histone deacetylase enzymatic activity to transcriptional repression by TGFβ is unknown. Here, the biological requirements for the interaction between Smads and endogenous histone deacetylase activity are investigated. We identify residues in Mad homology domain 1 of Smad3 that are required for association with histone deacetylase activity. An amino acid change at one of these critical residues does not disrupt the association of Smad3 with c-ski, SnoN, and transforming growth-interacting factor but does abrogate the ability of Smad3 to repress transcription. These findings indicate that the association of Smad3 and histone deacetylase activity relies on additional protein mediators that make contact with Smad3 at its amino terminus. Moreover, these data suggest that the suppressive effect of Smad3 on transcription is dependent upon its association with histone deacetylase enzymatic activity.

Transforming growth factor β (TGFβ)1 is a pleiotropic cyto-

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1 The abbreviations used are: TGFβ, transforming growth factor β; HDAC, histone deacetylase; MH, Mad homology; TGIF, transforming growth-interacting factor; DBD, Gal4 DNA binding domain; AD, Gal4 activation domain; SBE, Smad binding element; β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; FOA, 5-fluoroorotic acid; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione S-transferase; TNT, transcribed and translated; CMV, cytomegalovirus; CBP, CREB-response element-binding protein (CREB)-binding protein.
interactions between Smads and several HDAC1-associated proteins, TGIF, c-ski, and SnoN (28–33). Ectopic expression of TGIF, c-ski, or SnoN blocks the ability of TGFβ to modulate transcription. Furthermore, c-ski and TGIF were shown to compete with the co-activator, p300, for binding at the MH2 domain of Smad3. Therefore, the reported inhibitory effects of these HDAC1-associated proteins on TGFβ-activated transcription may result from competition between transcriptional co-activators and co-repressors for Smad binding.

Although these studies establish a physical link between the Smads and HDAC1, the contribution of Smad-mediated recruitment of histone deacetylase enzymatic activity to specific promoters has not been evaluated. To address this issue, we show that the ability of Smad3 to associate with endogenous HDAC enzymatic activity correlates with its ability to repress transcription. Using random mutagenesis and "reverse" two-hybrid analysis, a cluster of residues in the MH1 domain of Smad3 that are critical for the ability of Smad3 to associate with HDAC enzymatic activity are identified. A mutation at one of these residues does not affect the ability of Smad3 to activate transcription or to bind TGIF, c-ski, or SnoN in vitro. Surprisingly, this same mutation does abrogate the ability of Smad3 to repress transcription. Together, these results indicate that the association of Smad3 with histone deacetylase activity may require interactions with an unidentified HDAC-associated factor(s) at its amino terminus and that this association is critical for Smad3 to repress constitutive transcription. Moreover, these findings suggest that Smad3 may have distinct functional motifs associated with either the activation or repression of transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T and Mv1Lu cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin.

**Plasmids**—The 4×SBE-Lux reporter construct was made by annealing primers 5′-GATCTAATAGCTAGCAGGAGTGTAGC-3′ and 5′-GATGTCGATCTCCAGCTGACATCA-3′ and then concatenating them into the BglII site of pGL2-T+1 (34). HDAC1/pBSK was created by subcloning a PCR product derived from pCMV-FLAG-TGIF into the BamHI site of pBSK. Primers used to amplify the HDAC1 cDNA were as follows: 5′-CCGATGCTCCAGGAGATCGAGGAGAGATCC-3′ and 5′-CCGGATCTGCGGAGACTAAAGAAGAAG-3′. HA-c-ski/pBSK and HA-SnoN/pBSK were constructed by subcloning the EcoRI/XhoI restriction fragment of pCMV-FLAG-TGIF into the EcoRI/XhoI sites of pBSK. TGIF/pCAN Sin3A-pCS2 and SnoN/pCMV5b were subcloned into the BamHI site of pBSK. Primers used to amplify the HDAC1 cDNA were as follows: 5′-CCGATGCTCCAGGAGATCGAGGAGAGATCC-3′ and 5′-CCGGATCTGCGGAGACTAAAGAAGAAG-3′. HA-c-ski/pBSK and HA-SnoN/pBSK were constructed by subcloning the ClpuXbaI fragment of HA-c-ski/pCMV5b and HA-SnoN/pCMV5b into the ClpuXbaI sites in pBSK. TGIF/cAN was constructed by subcloning the EcoRI/XhoI restriction fragment of pCMV-FLAG-TGIF into the EcoRI/XhoI sites of pCAN (Oxyx Pharmaceuticals). Partial EcoRI/PvuII fragments of mutant Smad3 cDNAs in pBSK were subcloned into EcoRI/PvuII sites of Smad3/pRK5. Transfer of the mutant cDNAs was confirmed by sequencing. The Smad/SAM fragments of mutant Smad3 cDNAs in pRK5 were subcloned into pGEX-KG. Amino acids 1–147 encoding the DNA binding domain of Gal4 were subcloned into the HindIII-SalI site of Smad3/pRK5 (R. Derynick); pRK5Gal4 vector was constructed by replacing the Smad3-FLAG sequence in Smad3/pRK5 with the Gal4 DNA sequence. Smad3NL/pRK5Gal4, mutants of Smad3NL/pRK5Gal4, and Smad3C/pRK5Gal4 and were created by PCR using the primers 5′-TCTCCCCGATGGATCGTCCTCCATCGCGCTCTTC-3′ and 5′-CCGGATGCTCCAGGAGATCGAGGAGAGATCC-3′ and 5′-CCGGATCTGCGGAGACTAAAGAAGAAG-3′ to amplify cDNA from the appropriate Smad3/pRK5FLAG vectors. Each PCR product was subcloned into the Smad/UBam/Histone Deacetylase Activity to Repress Transcription

**PCR Mutagenesis**—The PCR mixture contained 100 ng of Smad3/pGBK7 DNA, 1 μM 5′-pGBK7 770 (5′-TCAGGGAGAGGAGGACTGATGCC-3′), 1 μM 3′ pGBK7 1077 (5′-GCTATACGCTGAGAAAGCAAC-3′), 1× PCR buffer (Life Technologies, Inc.), all four dNTPs (each at 50 μM), 0.1% Triton X-100, 1.5 mM MgCl2, 1 μg/μl bovine serum albumin, and 5 units of Taq DNA polymerase in 100 μl (1 min at 94 °C, 1 min at 45 °C, and 2 min at 72 °C for 40 cycles). 100 μM MnCl2 was added after 10 cycles. The PCR product was gel-purified.

**Reverse Two-hybrid**—Performed according to the protocol developed by Vidal et al. (see Ref. 36). The MaV103 strain of Saccharomyces cerevisiae carries the SPAL10::URA3 marker. Yeast two-hybrid protein-protein interactions confer sensitivity to 5-fluoroorotic acid (FOA) in yeast carrying this marker. A clonal line of MaV103 that expressed AD-349 isolated from the yeast two hybrid screen was transformed with 300 ng of the mutagenized Smad3 library and 150 ng of BamHI-linearized pGBK7. Several thousand transformants were first selected for growth in the presence of 0.1% FOA. These FOA-resistant transformants were tested for β-galactosidase (β-gal) activity using the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) filter lift assay (CLONTECH Matchmaker protocol). FOA-resistant transformants with lacZ reporter activity were then screened for less than wild type Smad3 were tested for Smad3 protein expression by Western analysis with anti-Gal4 DBD (Santa Cruz) monoclonal antibody. Clonal lines that expressed full-length, Smad3 fusion proteins at levels approximately equal to the wild type DBD-Smad3 expressing control line were considered positives. The Smad3 cDNAs from these lines were rescued and reintroduced with AD 349 into parental MaV103. Co-transformants were selected on synthetic dropout without tryptophan or leucine. Smad3 alleles with β-gal levels less than wild type Smad3 were sequenced.

**Histone Deacetylase Assays**—293T cells (∼5 × 10⁶) were transfected with 12 μg of pCGN vector or Smad/prRK5 constructs using a standard calcium phosphate protocol. 12 h post-transfection the cells were treated with 100 ng/ml trichostatin A (Wako BioProducts). 36 h post-transfection, the cells were lysed in LS (phosphate-buffered saline and 0.5% Nonidet P-40), 1 mM phenylmethylsulfonyl fluoride containing protease inhibitors. Lysates were immunoprecipitated with 1 μg anti-FLAG M2 (Eastman Kodak Co.) and protein A/G Sepharose (Amersham Pharmacia Biotech) as described. Immunoprecipitated complexes were washed 5 times with LS buffer containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitors and resuspended in HD buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol). Histone deacetylase assays were performed as described (37). Briefly, immunoprecipitated complexes were incubated for 30 min at 37 °C with 180 mg (150,000 dpm) of acid-soluble histones isolated from [3H]acetate-labeled chicken erythrocytes. Released [3H]acetate was extracted using ethyl acetate and quantified by scintillation counting.

**In Vitro Binding Studies**—Bacterially produced, glutathione-Sephrose-bound glutathione S-transferase (GST) and GST-Smad fusions were normalized for the amount of protein and volume of glutathione-Sepharose. These GST protein preparations were incubated with rabbit reticulocyte lysate in vitro transcribed translated (TNT) products (Promega) as described (35). TNT products were made using HDAC1/pBSK, HA-c-ski/pBSK, HA-SnoN/pBSK, TGIF/pCAN Sin3A-pCS2 + MT, and c-Jun/pBSK constructs as templates.

**Reporter Assays**—Luciferase assays were performed as previously described (39). LipofectAMINE (Life Technologies, Inc.) transfections were performed using the manufacturer's protocol. All transfections were normalized to β-galactosidase activity by co-transfection of 0.5 μg of a cytomegalovirus β-galactosidase (CMV-β-gal) expression vector. Twelve hours after transfection, 10 ng/ml TGFβ-1 in Dulbecco’s modified Eagle’s medium containing 0.1% fetal bovine serum was added. Luciferase activity was measured 20–24 h later. Chloramphenicol acetyltransferase assays were performed according to a standard protocol (39, 40). 293T cells were transfected for 7–12 h using calcium phosphate. 24 h later cells were assayed for chloramphenicol acetyltransferase activity.
RESULTS

The Smads Associate with HDAC1 in Yeast—To elucidate the mechanism of Smad-mediated transcriptional modulation of TGFβ-responsive genes, we set out to isolate transcription factors that bind to the Smads. A yeast two-hybrid screen of a human keratinocyte (HaCaT) cDNA library was performed using Smad3 as a bait. A cDNA clone (AD-349) encoding amino acids 211–482 of the histone deacetylase HDAC1 was isolated (Fig. 1A). Western analysis of yeast lysates confirmed that the expected protein product was produced (data not shown). To verify bait dependence of the interaction, AD-349 was reintroduced into the parental yeast line Hf7c in combination with expression vectors encoding DBD-Smad3 and either AD or AD-349, DBD-Smad4, and either AD or AD-349 and DBD with AD-349 according to the CLONTECH Matchmaker protocol. As a positive control for interaction, DBD fused to amino acids 72–390 of p53 (DBD-p53) and AD fused to amino acids 84–708 of SV40 large T antigen (AD-T antigen) were also included. Transformants were isolated on synthetic dropout media that lacks both tryptophan and leucine to select for cells carrying both a DBD fusion vector and the AD fusion vector, respectively. These cells were streaked onto synthetic dropout media that lacked tryptophan and leucine and either contained histidine. 5 mM 3-aminotriazole was introduced into Hf7c. Transformants were selected on synthetic dropout media lacking tryptophan and leucine.

Transformants were isolated on synthetic dropout media that lacked both tryptophan and leucine and either contained histidine. 5 mM 3-aminotriazole was included in the −histidine plate (see “Experimental Procedures”) WT, wild type.

![Cell Image](Image)

**Fig. 1. Smad3 and Smad4 associate with HDAC1 in yeast.** A, cDNA clone (349) encoding the carboxyl-terminal portion of the HDAC1 cDNA was isolated in a yeast two-hybrid screen using Smad3 as bait. A schematic depiction of clone 349 coding sequence compared with full-length HDAC1 is shown. B, Smad3 and Smad4 associated with clone 349 in *S. cerevisiae*. Protein interaction of DBD fusion proteins with AD fusion proteins activates the transcription of the HIS3 and lacZ marker genes in the *S. cerevisiae* strain, Hf7c. Here we show that the interaction of DBD-Smad3 or DBD-Smad4 with AD-349 activates the HIS3 reporter. Yeast strain Hf7c was transformed with the yeast expression vectors encoding DBD-Smad3 and either AD or AD-349, DBD-Smad4, and either AD or AD-349 and DBD with AD-349 according to the CLONTECH Matchmaker protocol. As a positive control for interaction, DBD fused to amino acids 72–390 of p53 (DBD-p53) and AD fused to amino acids 84–708 of SV40 large T antigen (AD-T antigen) were also included. Transformants were isolated on synthetic dropout media that lacks both tryptophan and leucine to select for cells carrying both a DBD fusion vector and the AD fusion vector, respectively. These cells were streaked onto synthetic dropout media that lacked tryptophan and leucine and either contained (+histidine) or lacked histidine (−histidine). 5 mM 3-aminotriazole was included in the −histidine plate (see “Experimental Procedures”) WT, wild type.
Smad3 Binds Histone Deacetylase Activity to Repress Transcription

We reasoned that the identification of amino acids in Smad3 required for its association with histone deacetylase activity would provide us with tools to dissect the role of histone deacetylases in Smad-directed transcription. To identify specific residues in Smad3 involved in the association between Smad3 and HDAC1, a reverse two-hybrid screen of a randomly mutagenized Smad3 library was performed. The entire Smad3 cDNA was mutated using random PCR mutagenesis. These PCR products were then introduced into a MaV103 yeast line expressing the AD-349 clone isolated in the screen for Smad3 binding partners (Fig. 1). The MaV103 yeast strain carries the SPAL10::URA3 marker in addition to the Gal4::lacZ and Gal4::HIS3 markers previously described (36). Yeast two-hybrid protein-protein interactions confer sensitivity to FOA in lines carrying this reporter. Several thousand transformants were screened for their ability to grow in the presence of 0.1% FOA and their inability to produce a blue color in the presence of X-gal. From this set of positives, clones that expressed full-length DBD-Smad3 proteins to a level similar to the wild type Smad3 line were selected. In all, nine clones met the selection criteria. A map of these mutants is depicted in Fig. 3. As a measure of lacZ reporter gene activity, the level of β-gal activity produced by yeast expressing AD-349 and each Smad3 mutant is shown.

Confirming the validity of the screen, β-gal activity for each Smad3 mutant allele was significantly reduced compared with that of wild type. Although reporter gene activity for some of the mutations is approximately half that of wild type Smad3, other mutant alleles, particularly L38P and N123D/I298M, give β-gal activities comparable with negative controls for the interaction. Notably, all of the clones contain a missense mutation in the MH1 domain of Smad3, suggesting that the MH1 domain may make contact with HDAC1-binding proteins. One particular amino acid, Tyr-127, was mutated in two different clones, highlighting its importance for the interaction. Two other mutants, Smad3F111S/W394R and Smad3N123D/I298M, contain two missense mutations, one in the MH1 domain and one in the MH2 domain. A subset of these mutations displaying the lowest β-gal activities was subcloned into mammalian expression vectors to be studied further.

Characterization of Smad3 HDAC Activity Mutants—Given that the yeast two-hybrid interaction between Smad3 and HDAC1 may be dependent upon yeast proteins, the HDAC1-containing complexes that associate with Smad3 in our yeast two-hybrid interaction may be very different from those that associate with Smad3 in mammalian cells. To determine if these mutations have any effect on the association of Smad3 with endogenous histone deacetylases in human cells, we measured the level of histone deacetylase activity associated with individual mutants in 293T cells (Fig. 4A). Approximately equal amounts of wild type and mutant Smad3-FLAG fusion proteins were immunoprecipitated, suggesting that each mutant is stable in mammalian cells. In this assay, each mutant was significantly deficient in its ability to associate with HDAC activity, demonstrating the importance of these particular MH1 domain residues in the association of active histone deacetylases with Smad3.

Similar assays using Smad3 deletion mutants confirmed the importance of the MH1 domain for the coupling of HDAC activity with Smad3. Smad3NL, Smad3C, and Smad3ΔC were overexpressed, immunoprecipitated, and analyzed for HDAC activity (Fig. 4, B and C). Strikingly, Smad3NL associated with 3-fold more HDAC activity than full-length Smad3. The level of HDAC activity associated with Smad3NL was comparable with Sin3A-bound HDAC activity, confirming that Smad3 binds biologically relevant levels of HDAC activity through its MH1 domain. Smad3ΔC co-immunoprecipitated with levels of associated HDAC activity similar to full-length Smad3. In contrast, Smad3C associated with little or no HDAC enzymatic activity.

Examination of the crystal structure of Smad3 MH1 domain revealed that many of these mutations alter amino acids in the hydrophobic core of the protein (52). Thus, some of these amino acid changes may lead to gross conformational changes in the MH1 domain that block several Smad3 functions such as DNA binding, nuclear entry, Smad4 binding, and transcriptional activation. To address these issues, the ability of Smad3 and the Smad3 mutants to activate the 4xSBE reporter was assessed (Fig. 5A). This reporter contains Smad consensus DNA binding sites (SBEs) to which Smad3 and Smad4 directly bind.
and activate transcription. In this assay, the ability of mutant L38P to activate transcription was severely inhibited. In contrast, F111S/W394R, Y125C, and Y127C activated transcription to levels similar to those of wild type Smad3. Activation of transcription by P95S and N123D/I298M was partially hindered. Mutated amino acids that are accessible to the surface of the wild type protein are noted (Fig. 5B). Taken together, these data suggest that unlike other mutations isolated from the reverse two-hybrid screen, the F111S and Y125C mutations might disrupt a discreet binding surface for HDAC1-binding proteins without inhibiting the DNA binding, nuclear entry, Smad4 cooperation, and transcriptional activation functions of HDAC1.
Smad3. Supporting this notion, the MH1 domains of bacterially produced GST-Smad3NL-F111S and GST-Smad3NL-Y125C retain the ability to directly associate with the SBE by electrophoretic mobility shift assay (data not shown).

c-ski, SnoN, and TGFβ have been shown to mediate the association of specific Smads with HDAC1. The inability of the mutant clones derived from the reverse two-hybrid screen to associate with HDAC activity in mammalian cells may be the result of deficient association with these co-repressor proteins. To address this issue, the ability of in vitro TNT c-ski, SnoN, and TGFβ to bind GST fusion proteins of Smad3, Smad3F111S/W394R, and Smad3Y125C was assessed (Fig. 5C). The inability of GST-Smad3 to bind TNT-HDAC1 was mentioned previously and is shown here as a negative control for the binding assays. As a positive control, the results of in vitro binding reactions between each Smad3 mutant and TNT-c-Jun is shown (35, 53).

c-Jun forms critical contacts with lysine 41 in the MH1 domain of Smad3 (54). Although Smad3F111S/W394R bound TNT-c-Jun with similar efficiency as wild type Smad3, consistently the association of Smad3Y125C and TNT-c-Jun was slightly diminished. Therefore, at least under in vitro conditions, the Y125C mutation may cause minor alterations to overall protein structure rather than solely affecting a discreet surface. Consistent with this observation, the ability of Smad3Y125C to associate with other TNT products was slightly diminished. In contrast, no consistent difference between the affinities of Smad3 and Smad3F111S/W394R for TNT-c-ski, TNT-SnoN, and TNT-TGFβ was observed, indicating that direct binding to c-ski, SnoN, and TGFβ is unaffected by this mutation.

Transcriptional Repression by Smad3 HDAC Activity Mutants Is Deficient—Based on evidence showing a connection between HDAC activity and the repression of transcription, we postulated that the ability of the Smads to associate with HDAC activity would allow them to repress constitutive transcription. To quantify Smad-mediated repression, a reporter construct containing GAL4 DNA binding sites upstream of the constitutively active SV40 promoter was employed (Fig. 6A).

Previous reports show that Gal4 fusions of the transcriptional repressors, Mad1 and Rb, inhibit the expression of this same reporter (55, 56). In this assay, Smad3 and Smad4 significantly repressed transcription when tethered to DNA (Fig. 6, B and C, and data not shown). Reporter activity was repressed ~8-fold further by Gal4-Smad3NL but was unaffected by Gal4-Smad3C expression.

If the ability of Smad3NL to repress transcription was indeed dependent upon its association with HDAC enzymatic activity, we predicted that repression of reporter gene expression by Smad3 HDAC activity mutants would be deficient. To address this, the effect of the NL domains of the Smad3Y125C and Smad3F111S HDAC activity mutants on reporter gene activity was assessed (Fig. 6D). Consistent with a role for the Smad3-HDAC interaction in the repression of active transcription, the ability of each mutant to repress reporter gene expression was significantly inhibited compared with wild type Smad3NL. In contrast, the expression level of each mutant was similar to wild type Smad3 NL (Fig. 6E).

DISCUSSION

The ability of TGFβ to repress the expression of critical cell cycle regulators like c-Myc indicates that transcriptional repression by TGFβ may contribute to TGFβ-mediated inhibition of cellular proliferation. Thus, understanding the mechanisms through which TGFβ represses transcription may provide insight into the anti-proliferative capacity of TGFβ and the role of Smads in tumor suppression. To this end, we show that Smad1, Smad2, Smad3, and Smad4 associate with endogenous histone deacetylase activity. Thus, Smad-HDAC complexes are enzymatically active and may couple histone deacetylase activity with nuclear Smad activities like DNA binding. A mutant lacking the MH2 domain, Smad3NL, associated with 3-fold more histone deacetylase enzymatic activity than Smad3. The amount of Smad3NL-associated activity is comparable with the level of HDAC activity that co-immunoprecipitated with the transcriptional repressor and direct HDAC1-binding protein, Sin3A. Thus, Smad3 associates with levels of HDAC activity that are biologically significant in the repression of transcription.

The difference in HDAC activity isolated from Smad3 full-length and Smad3NL immunoprecipitations suggests that the MH2 domain may inhibit the association of histone deacetylases with full-length Smad3. A previously characterized autoinhibitory interaction between the MH1 and MH2 domains may be responsible for this effect. Activation of Smad3 by TGFβ is thought to interrupt the binding of the MH1 and MH2
domains to each other and may facilitate the association of Smads with HDAC-binding proteins (57). Alternatively, histone acetyltransferase activity of p300/CBP bound at the MH2 domain is inhibited.

In vitro binding of TGIF, c-ski, and SnoN to the HDAC activity mutant identified in these studies, Smad3F111S/W394R, was essentially unaffected. It is possible that, TGIF, c-ski, and SnoN are not present in these complexes, but they do make up and may be essential for the formation of other Smad-HDAC complexes not isolated or characterized in these assays. Supporting this possibility, our Smad3 HDAC activity mutants were based on an interaction between Smad3 and HDAC1 in S. cerevisiae, an organism with no homologues to TGIF, c-ski, or SnoN. Furthermore, previous studies show that the Smad MH2 domain rather than the MH1 domain makes contact with c-ski and SnoN (29–32, 63, 64). We demonstrate here, however, that Smad3NL, a Smad3 fragment lacking the MH2 domain, associates with biologically relevant levels of HDAC enzymatic activity.

Alternatively, TGIF, c-ski, and SnoN do participate in the HDAC complexes isolated in the studies presented here, but their association with Smad3 is not sufficient to mediate the interaction between Smad3 and HDAC activity when HDAC binding at the MH1 domain is blocked. In this scenario, TGIF, c-ski, and SnoN association at the MH2 domain may serve to stabilize the essential MH1 interaction. Competition with p300 for binding at the MH2 domain may have masked this role of TGIF, c-ski, and SnoN in the HDAC activity assays reported here. Indeed, previous reports show that p300/CBP competes with TGIF and c-ski for Smad binding (32, 65). If p300/CBP does compete with TGIF, c-ski, and SnoN in our studies, we predict that inhibition of p300 binding in these assays may result in the co-immunoprecipitation of more HDAC activity with Smad3 and Smad3Δc. In contrast, a block of Smad3C/p300 association is not expected to elevate HDAC activity levels associated with Smad3C since the MH1 domain residues essential for the interaction between Smads and HDAC activity are not present in this particular mutant.

In the transcriptional assay used here, the Smads are tethered to a viral promoter that is constitutively transcribed. We show that Smad3 represses constitutive transcription from this promoter. Inconsistent with previous reports that Smad-activated transcription is blocked by Smad binding partners, transcriptional repression by Smad3 in this assay was independent of TGFB-activated or Smad-activated transcription (28, 30–32, 64, 66). In the context of this assay, transcriptional repression by Smad3F111S and Smad3Y125C was significantly abrogated compared with wild type Smad3. Consistent with these findings, Smad3C, which did not associate with HDAC activity, did not repress transcription. Together, these data suggest that, in the context of this assay, the HDAC enzymatic activity of the Smad3-HDAC complex is required for the repression of constitutive transcription by Smad3.

The ability of the Smad3 HDAC-activity mutants to repress transcriptional repression by Smad3 in this assay was independent of TGFB-activated or Smad-activated transcription (28, 30–32, 64, 66). In the context of this assay, transcriptional repression by Smad3F111S and Smad3Y125C was significantly abrogated compared with wild type Smad3. Consistent with these findings, Smad3C, which did not associate with HDAC activity, did not repress transcription. Together, these data suggest that, in the context of this assay, the HDAC enzymatic activity of the Smad3-HDAC complex is required for the repression of constitutive transcription by Smad3.
transcription was, however, partially retained. This may simply reflect the nature of the SV40 promoter that is sensitive to HDAC-independent modes of repression (56). Thus, a portion of the inhibitory effect of Smad3 on reporter gene activity may be independent of HDACs, suggesting that in addition to the recruitment of histone deacetylase activity to a particular promoter, the Smads may repress constitutive transcription through HDAC-independent mechanisms. Nevertheless, a significant portion of Smad3-mediated transcriptional repression found here is dependent upon the association of Smad3 with histone deacetylase activity. The finding that the repression-deficient mutants, Smad3F111S/W394R and Smad3Y125C, retain the ability to activate 4xSBE transcription indicates that the activation and repressive effects of Smad3 on transcription may exist as two separable functions of the Smad3 protein. We envision a model where, under specific promoter contexts, association of Smad3 and p300/CBP is favored and transcription is activated. The competition between p300/CBP and HDAC-association of Smad3 and p300/CBP is favored and transcription is activated similarly by wild type Smad3, and each associated repressors would determine the extent of transcriptional activation and repressive effects of Smad3 on transcription. The inhibitory effect of Smad3 on reporter gene activity may be inferred a model where, under specific promoter contexts, as-
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