Novel Mutations in Smad Proteins That Inhibit Signaling by the Transforming Growth Factor β in Mammalian Cells[†]

Vassiliki Prokova,[‡,#] Sofia Mavridou,[‡,#] Paraskevi Papakosta,[‡] Kyriacos Petratos,[§] and Dimitris Kardassis*[,‡,||]

Laboratory of Biochemistry, Department of Basic Sciences, University of Crete Medical School, Heraklion 71110, Greece, and the Protein Structure and Function Group and Gene Expression Group, Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology-Hellas, Heraklion 71110, Greece

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ABSTRACT: Smad proteins are the key effectors of the transforming growth factor β (TGFβ) signaling pathway in mammalian cells. The importance of Smads for human physiology is documented by the identification and characterization of mutations that are frequently found in cancer patients. In the present study we have functionally characterized such a tumorigenic mutation in Smad4 (E330A) and shown that this mutant as well as a Smad3 mutant bearing the corresponding mutation (Smad3 E239A) failed to activate transcription in response to TGFβ stimulation because of defects in homo-and hetero-oligomerization. In the case of Smad3, the E239A mutation also abolished the phosphorylation by the TGFβ type I receptor (ALK5). Examination of the previously published crystal structure of a Smad3/Smad4 MH2 heterotrimer [Protein Data Bank accession code, 1U7F] showed that (a) residue E239 in Smad3 participates in a dense network of intermolecular hydrogen bond and ionic interactions with other conserved polar residues such as Y237 of β1 strand, N276 and R279 of L2 loop, and R287 of helix H1; (b) residue R287 in Smad3 is also involved in intermolecular interactions by making hydrogen and ionic bonds with Y364 in Smad3 and D493 in Smad4, an amino acid residue that is also frequently mutated in cancer patients (mutation D493H). To investigate the contribution of these interactions to Smad function and TGFβ signaling, we replaced two of these polar residues (R287 and Y237) with a nonpolar residue (alanine) and functionally characterized the resulting Smad3 mutants. Our analysis showed that Smad3 mutant R287A was phosphorylated by the ALK5 receptor but was unable to form homo-oligomers or hetero-oligomers with Smad4 and activate transcription whereas mutation Y237A had a wild type phenotype. In summary, our present work provides a molecular basis for the functional inactivation of the TGFβ pathway in patients bearing previously uncharacterized mutations in Smad4 as well as new information regarding the importance of conserved polar amino acids for the structure and function of the MH2 domain of Smads.

Members of the transforming growth factor β (TGFβ[1]) superfamily of cytokines control important biological processes including cell proliferation, differentiation, apoptosis, angiogenesis, lineage determination, motility, adhesion, and epithelial to mesenchymal transdifferentiation (EMT) in a cell type-dependent manner (*1*, *2*). The interaction of TGFβ with its receptors (type I and type II Ser/Thr kinase receptors) on the cell surface is followed by the phosphorylation of a subset of intracellular signaling mediators termed receptor-regulated Smads (R-Smads) (*3*−*7*). The phosphorylation of R-Smads (Smad 2 and 3) by the type I TGFβ receptor induces their oligomerization with the common mediator Smad4 and their translocation to the nucleus where they bind to the promoters of target genes and activate or repress their transcription (*3*−*7*). To accomplish this task, R-Smad/Smad4 complexes in the nucleus cooperate with DNA-bound co-factors and non-DNA bound coactivators and corepressors (*3*−*7*).

Smads harbor two well conserved functional domains of defined structure termed the Mad homology 1 (MH1) and MH2 domain separated by a middle nonconserved flexible linker region (*7*). The MH1 domain is responsible for binding to DNA by making contacts with nucleotides in Smad DNA binding elements (SBEs) composed of the tetranucleotide 5′-GTCT-3′ (*8*). The MH1 domain contains a nuclear

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* Address correspondence to: Dimitris Kardassis, Department of Basic Sciences, University of Crete Medical School, Heraklion, Crete, Greece GR-71110, Tel: +30-2810-394549, Fax: +30-2810-394530, E-mail: kardasis@imbb.forth.gr.

[‡] University of Crete Medical School.

[§] Protein Structure and Function Group, Foundation of Research and Technology-Hellas.

[||] Gene Expression Group, Foundation of Research and Technology-Hellas.

[#] These authors have contributed equally to this work.

[1] Abbreviations: ALK5, activin receptor like kinase type 5; BMP, bone morphogenetic protein; DBD, DNA binding domain; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HEK, human embryonic kidney; HRP, horseradish peroxidase; MH1, Mad homology domain 1; p/CAF, p300/CBP activating factor; PDB, Protein Data Bank; PCR, polymerase chain reaction; R-Smads, receptor-regulated Smads; SBEs, Smad DNA binding elements; TGFβ, transforming growth factor β; WB, western blotting; wt, wild type; WCE, whole cell extracts.

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localization signal and negatively regulates the functions of the MH2 domain (*9*, *10*). The MH2 domain is indispensable for Smad homo- and hetero-oligomerization, for the recognition and phosphorylation of R-Smads by their type I receptors, and for transcriptional activation (*7*). The key structural feature of the MH2 domain is a central β-sandwich core element capped by a three-helical bundle at one end and a loop-helix region at the opposite end (*11*−*15*). Smad oligomerization is facilitated by specific contacts between amino acids of the loop-helix region of one MH2 subunit and the three helical bundle of another partner subunit (*11*). Point mutations in highly conserved amino acid residues either in the loop-helix region or the three-helical bundle of the MH2 domain that disrupt Smad4 homo-oligomerization have been described in cancer patients (*12*, *16*).

We have shown recently that TGFβ-stimulated transcriptional activation of target genes via Smad3 protein is mediated by two distinct transactivation domains present in the C-terminal MH2 domain and the middle linker region (*17*). We characterized the linker region of Smad3 further and showed that it has the capacity to physically and functionally interact with the histone acetyltransferase p300/CBP activating factor (p/CAF) in a ligand-independent manner and to be transcriptionally active in yeast cells that lack a TGFβ pathway (*17*). In the same study, we showed that the transcriptional activity of the linker depends on the presence of a short region that includes the first and part of the second β strands of the MH2 domain (aa region 230−248) (*17*).

In the present study, we continue our structure−function analysis of Smad proteins by functionally characterizing naturally occurring tumorigenic mutations as well as novel bioengineered mutations. Our aim was (a) to gather new information regarding to the contribution of specific structural elements of Smads to their functions as TGFβ signaling mediators; (b) to provide a molecular basis for the functional inactivation of the TGFβ pathway in patients bearing previously uncharacterized mutations in specific polar amino acid residues of Smad4.

MATERIALS AND METHODS

Materials. Dulbecco's Modified Eagle's Medium (DMEM) and penicillin/streptomycin for cell culture was purchased from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Restriction enzymes and modifying enzymes (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). GoTaq DNA polymerase, dNTPs, the luciferase assay system, and Wizard SV gel and PCR cleanup system were purchased from Promega (Madison, WI). Streptavidin−agarose, streptavidin−HRP, and the anti-Flag M2 mouse monoclonal antibody were purchased from Sigma-Aldrich (St Louis, MI). The Super Signal West Pico Chemiluminescent Substrate was purchased from Pierce (Rockford, IL). The mouse anti-GAL4 DBD antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-myc monoclonal antibody was a kind gift from Dr. G. Mavrothalassitis (University of Crete, Heraklion, Greece). The anti-Smad3 and anti-P-Smad3 antibodies were provided by Dr. Aris Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). The anti-mouse peroxidase conjugated secondary antibody was purchased from Chemicon International Inc. (Temecula, CA).

Plasmid Constructions. Plasmids expressing the wt human Smad3 tagged with the myc epitope or fused with the DNA binding domain (DBD) of GAL4 have been described previously (*17*, *18*). The mutants bearing the internal deletion of amino acids 230−248 or the single amino acid substitutions E239A, Y237A, and R287A in Smad3 as well as the E330A mutation in Smad4 were constructed by overlap extension PCR (*19*). Each amplified fragment corresponding to a particular Smad mutant was cloned at the EcoRI and NotI sites of either the pCDNA1amp-6Xmyc vector in frame with a 6myc epitope tag or into the pCDNA3-Bio vector in frame with the biotinylation tag and then subcloned into the pBXG1 vector at the EcoRI and XbaI sites in frame with the DNA binding domain of GAL4. The sequences of all primers used in the PCR amplifications are shown in Table 1 (Supporting Information). All mutant Smad3 cDNAs were sequenced for verification and found to contain the proper mutation. Plasmid pBS-myc-BirA bearing the myc-tagged bacterial biotin ligase BirA was a generous gift of Dr. John Strouboulis (Erasmus Medical Center, Rotterdam, The Netherlands). The expression vector pCDNA3-Bio has been described previously (*17*, *20*).

Cell Culture and Treatments. COS-7, HepG2, HEK-293T, JEG-3, and MDA-MB-468 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and penicillin−streptomycin, in a 37 °C, 5% CO_2 incubator. Treatment with 200 pM of TGFβ1 was for 24 h.

Transient Transfections and Reporter Assays. Transient transfections for transactivation assays were performed by the calcium phosphate coprecipitation method in six-well plates using 1 μg of a reporter plasmid and 1−2 μg of expression vectors per well. For immunofluorescence analysis, 5×10^5 COS-7 cells were transfected in 6-cm plates with 2 μg of each 6-myc Smad expression vector in the presence or in the absence of a constitutively active TGFβ type I receptor (2 μg). β-Galactosidase and luciferase assays were performed using well-established protocols.

Indirect Immunofluorescence. Transfected COS-7 cells were seeded on glass coverslips, 22 × 22 mm, covered with 0.1% gelatin and incubated for 16−18 h. Cells were washed three times on a slow rotating platform with PBS+/+ (PBS plus 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and fixed with 3% *p*-formaldehyde in PBS +/+ for 5 min at room temperature. Cells were washed three times with PBS+/+ and permeabilized with 0.5% Triton X-100 in buffer 1 (10× buffer 1: 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.5 mM glucose, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, 20 mM MES, pH 6.0−6.5) for 5 min at room temperature. Cells were washed three times with PBS+/+ and blocked with PBS +/+/1.5% FBS two times. Cells were incubated with anti-myc (9E10), 1:200 dilution in PBS +/+/1.5% FBS for 30 min at 4 °C. Cells were washed three times with PBS+/+ /1.5% FBS and incubated with the secondary antibody (goat anti-mouse FITC, 1:50 dilution in PBS +/+/1.5% FBS) for 30 min at 4 °C in the dark. Cells were washed three times with PBS+/+ in the dark and mounted on glass slides using mounting solution

(1:1 glycerol/PBS). Cells were observed using a Leica SP confocal fluorescent microscopy.

Western Blot Analysis. Cell lysates or proteins bound to streptavidin agarose beads were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Life Sciences and Schleichers & Schuell), with a Bio-Rad Protean electroblot apparatus. Electrophoresis was performed on 10.5% polyacrylamide gel electrophoresis in 500 mL of 1× TGS (1 L of 10× TGS: 30.3 g of Tris, 144.2 g of glycine, 10 g of SDS, pH 8.3). Proteins on the membrane were visualized by Ponceau S staining. Nitrocellulose membranes were washed with TBS-T (TBS + 0.05% Tween-20) for 10 min, at room temperature. Nonspecific sites were blocked by soaking the membrane in TBB buffer (1× TBS + 5% nonfat milk or 5% BSA, 0.1% Tween-20) for 2 h at 4 °C. Western blotting was performed with a 1:5000 dilution of the anti-myc or the anti-FLAG M2 monoclonal antibodies, 1:1000 dilution of the anti-GAL4 (DBD) monoclonal antibody or the anti-P-Smad3 antibody and 1:400 dilution of the anti-Smad3 antibody in TBB overnight at 4 °C. The membranes were washed three times with TBS-T, for 10 min, at room temperature. As a secondary antibody we used anti-mouse or anti-rabbit horseradish peroxidase-conjugated (HRP), in a 1:10 000 dilution in TBS-T, for 1 h at room temperature. In the case of biotinylated proteins, membranes were hybridized directly with HRP-conjugated streptavidin in a 1:10 000 dilution for 1 h at room temperature. After three washes of 15 min with TBS-T at room temperature, bands were visualized by enhanced chemiluminescent detection on Fuji medical X-ray film (Super RX).

In Vivo Biotinylation and Protein−Protein Interaction Assay. For the in vivo biotinylation assay (*21*), 7.5×10^5 of HEK-293T cells were transfected in 10-cm dishes with 7.5 μg of the pCDNA3-Bio-Smad3 expression vector in the presence or in the absence of 7.5 μg of pCDNA3-BirA vector expressing the bacterial biotin ligase BirA. For protein−protein interaction assays, HEK-293T cells were cotransfected with the above plasmids along with 7.5 μg of expression vectors pCDNA3-6myc-Smad2, pCDNA3-6myc-Smad3, pCDNA3-6myc-Smad4, pCDNA3-flag-p/CAF, or pCDNA3-flag-c-Ski in the presence or in the absence of an expression vector for a constitutively active form of the type I TGFβ receptor (ALK5-ca, 7.5 μg). Cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100) and allowed to interact with streptavidin agarose beads for 3 h at 4 °C in a rotating platform. Beads were washed three times with lysis buffer and centrifuged at 4000 rpm for 1 min at room temperature. Bound proteins as well as the starting material (input) were subjected to SDS-PAGE followed by immunoblotting as described above.

RESULTS

An Internal Deletion of the 230−248 Region of Smad3 Abolished Its Function as a TGFβ Signaling Mediator. We have shown previously that TGFβ-stimulated transcriptional activation of target genes via Smad3 protein is mediated by two distinct transactivation domains present in the C-terminal MH2 domain and the middle linker region and that a small region in the junction between these two domains (aa 230−248) is essential for the activity of both domains (*17*). To investigate further the mechanisms by which the 230−248 region of Smad3 contributes to its functions as a TGFβ signaling mediator, we constructed a new Smad3 mutant bearing an internal deletion of this region (Smad3 Δ230−248, Figure 1A). The transcriptional activity of the new Smad3 mutant was first evaluated by transactivation experiments in the human JEG-3 choriocarcinoma cell line which does not express endogenous Smad3 protein (*22*). For this purpose, a synthetic Smad-dependent promoter consisting of 12 tandem Smad binding elements ($CAGA_{12}$) and the minimal E1B promoter was utilized. As shown in Figure 1C, a constitutively active type I TGFβ receptor (CA-ALK5) could not activate this promoter in JEG-3 cells because of the absence of endogenous Smad3. Exogenous expression of 6myc-tagged wild type Smad3 protein in these cells rescued the TGFβ signaling pathway and transactivated the promoter in the absence, and more strongly in the presence, of CA-ALK5. In contrast, no promoter transactivation was osberved when JEG-3 cells were transfected with an expression vector for the Smad3 mutant Δ230−248 either in the absence or in the presence of the CA-ALK5 receptor (Figure 1C). By increasing the concentration of the Smad3 Δ230−248 expression vector several fold, we failed to activate the $(CAGA)_{12}$E1B promoter above the background levels suggesting that this internal deletion strongly affected the function of Smad3 as a TGFβ-inducible transcriptional activator (data not shown).

The transcriptional activity of the Smad3 Δ230−248 mutant was also examined in the breast cancer cell line MDA-MB-468 that lacks endogenous Smad4 because of a homozygous deletion of the Smad4 gene (*23*, *24*). As shown in Figure 1D, a strong transcriptional activation of the $(CAGA)_{12}$-E1B promoter was achieved by the simultaneous expression of wild type Smad3 and Smad4 proteins indicative of a strong transcriptional cooperativity between the two TGFβ signaling mediators. In contrast, the Smad3 Δ230−248 mutant failed to functionally cooperate with Smad4 either in the absence or in the presence of TGFβ (Figure 1D). Similar results were obtained using a human hepatoma cell line (HepG2) which expresses both Smad3 and Smad4 proteins endogenously (Figure 1E). The expression of the Smad3 (Δ230−248) mutant was verified by immunoblotting analysis (Figure 1B).

The ability of the Smad3 mutant bearing the internal deletion Δ230−248 to activate transcription was also examined using the GAL4 transactivation assay. For this purpose, the cDNAs of wild type Smad3 or the Smad3 Δ230−248 mutant were cloned in frame with the DNA binding domain of GAL4 (Supporting Information Figure 1A). Immunoblotting analysis confirmed the expression of the GAL4-Smad3 Δ230−248 mutant compared to wild type GAL4-Smad3 in HepG2 cells (Supporting Information Figure 1B). As shown in Supporting Information Figure 1C, wild type GAL4-Smad3 protein strongly enhanced the activity of the GAL4-responsive promoter G5-E1B in HepG2 cells, and this transactivation was further enhanced by the constitutively active type I TGFβ receptor (CA-ALK5). In contrast, the GAL4-Smad3 Δ230−248 mutant was transcriptionally silent either in the absence or in the presence of CA-ALK5 (Supporting Information Figure 1C).

Using the same system, we showed that exogenous expression of wild type Smad4 in MDA-MB-468 Smad4

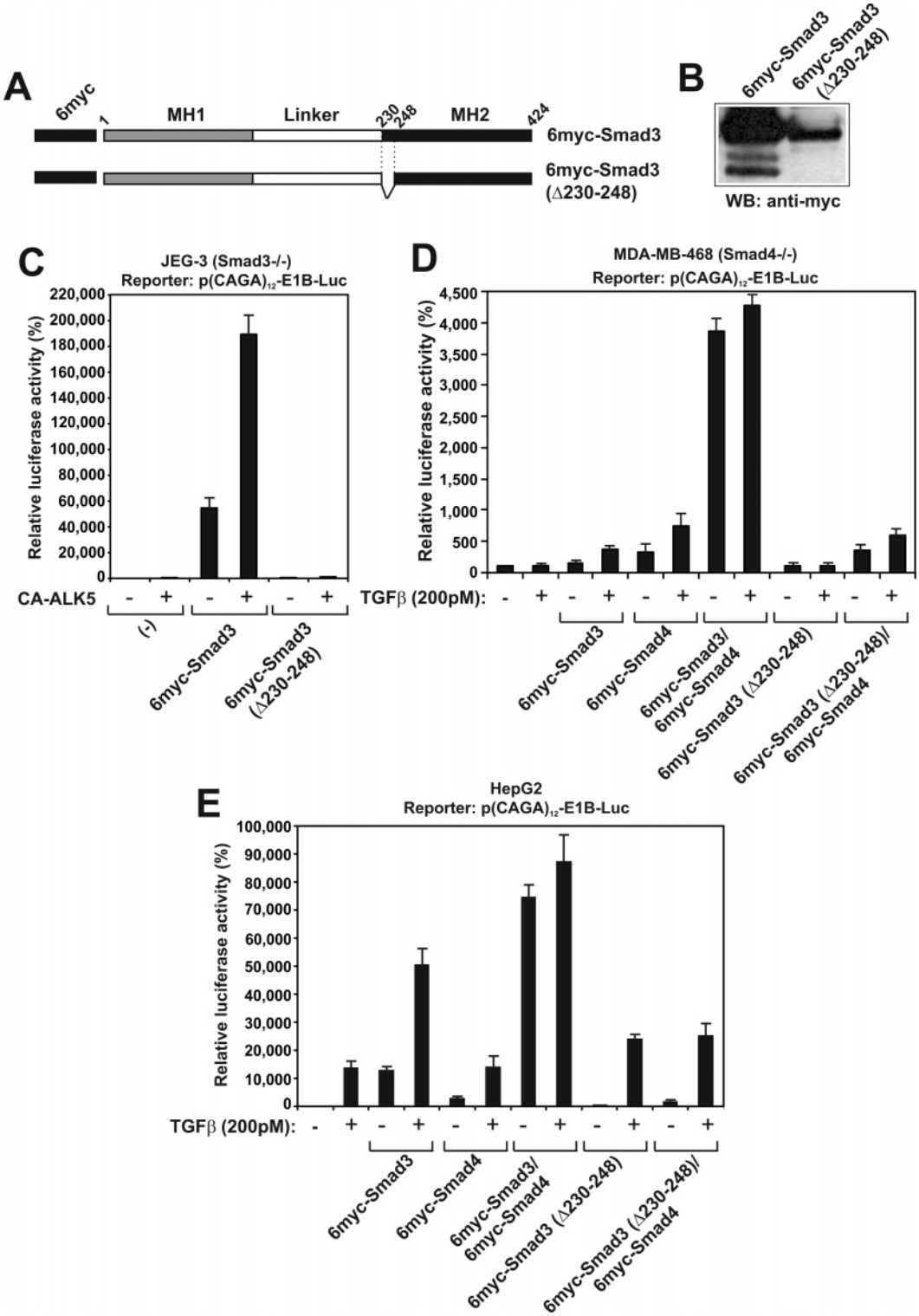

FIGURE 1: A−E: Deletion of the 230−248 region abolished the transcriptional activity of Smad3 protein and its functional cooperation with Smad4. Panel A, Schematic representation of wild type Smad3 and the Smad3 Δ230−248 mutant that were utilized in the experiments of panels C, D, and E. Vertical dashed lines and numbers show the coordinates of the deletion. Panel B, Relative levels of expression of wild type Smad3 and the Δ230−248 mutant. HEK293T cells were transfected with equal amounts of expression vectors of 6myc-Smad3 and 6myc-Smad3 (Δ230−248). Equal amounts of whole cell extracts from the transfected cells were subjected to SDS-PAGE and immunoblotting using the anti-myc antibody. Panel C, Human choriocarcinoma JEG-3 cells were transfected with 1 μg of expression vectors for 6myc-Smad3 or 6myc-Smad3 (Δ230−248) in the absence or in the presence of an expression vector for CA-ALK5 (1 μg) along with the $p(CAGA)_{12}$-E1B-Luc reporter (1 μg). The normalized mean values of luciferase activity (±SEM) are shown with a histogram. Panels D and E, Breast cancer MDA-MB-468 cells (Panel D) or HepG2 cells (Panel E) were transfected with the $p(CAGA)_{12}$-E1B-Luc reporter (1 μg) along with expression vectors for 6myc-Smad3, 6myc-Smad3 (Δ230−248), and 6myc-Smad4 (1 μg) in the combinations shown at the bottom of the graph and were either treated with TGFβ (200 pM) for 24 h (+) or left untreated (−). The normalized mean values of luciferase activity (±SEM) are shown with histograms. In Panels C, D, and E, all values are shown as % relative luciferase activity relative to the reporter $(CAGA)_{12}$-E1B-Luc alone, the activity of which was taken as 100%.

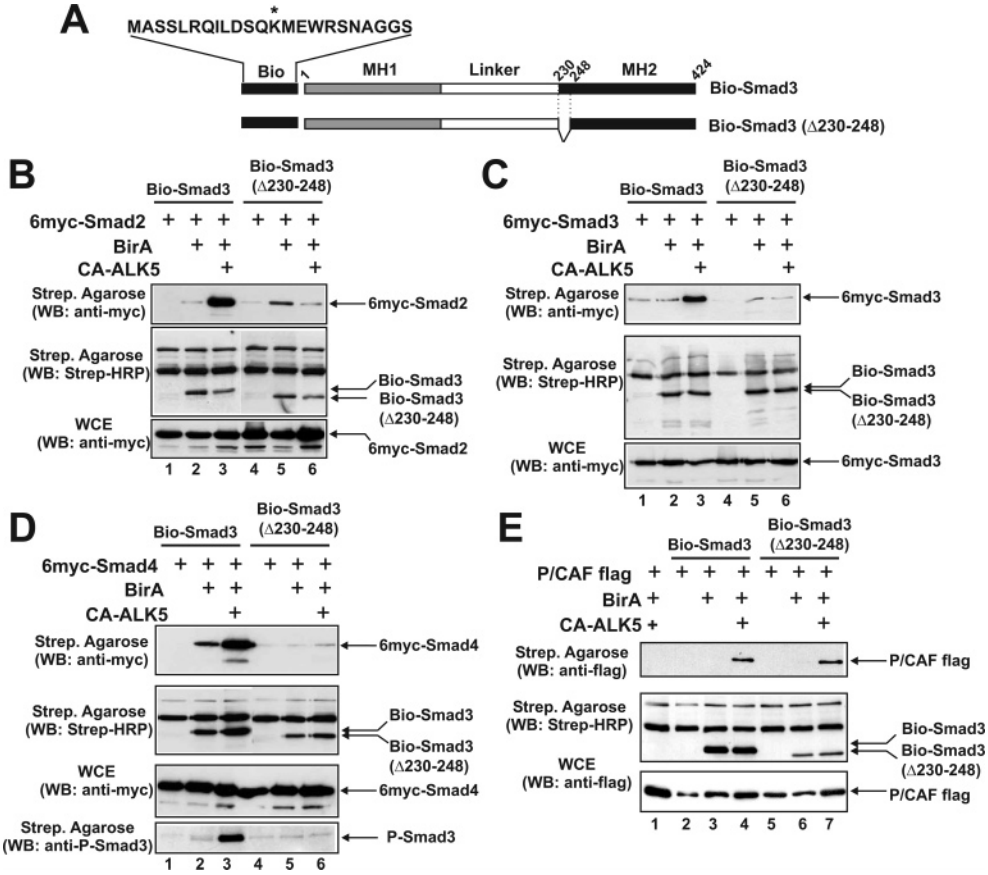

FIGURE 2: A−E: Utilization of an in vivo biotinylation assay to monitor the homo- and hetero-oligomerization properties of the wild type and the Δ230−248 mutant Smad3 proteins: Panel A, Schematic representation of wild type Smad3 and the Smad3 Δ230−248 mutant bearing, at their N-terminus, the 23 amino acid peptide tag (Bio) that serves as a target of biotinylation by the biotin ligase BirA. The lysine residue that is biotinylated in the presence of BirA is indicated with an asterisk. Panels B, C, and D, HEK-293T cells were transfected with expression vectors for Bio-Smad3 or Bio-Smad3 Δ230−248 along with 6myc-Smad2 (Panel B), 6myc-Smad3 (Panel C), and 6myc-Smad4 (Panel D) in the absence or in the presence of BirA and CA-ALK5 as indicated on top of each panel. The concentrations of plasmids used and the protocol of the protein−protein interaction assay are described in detail in Materials and Methods. Western blottings were performed using the anti-myc monoclonal antibody for the detection of myc-tagged Smad2, Smad3, and Smad4 proteins, horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad3 proteins, and the anti-phospho Smad3 antibody to detect phosphorylated Smad3 (P-Smad3). The arrows on the right show the position of the indicated proteins. WCE: whole cell extract. Strep: streptavidin. HRP: horseradish peroxidase. Panel E, The Δ230−248 deletion does not affect the CA-ALK5-dependent association of Smad3 with the coactivator/histone acetyltransferase p/CAF: HEK-293T cells were transfected with expression vectors for Bio-Smad3 or Bio-Smad3 Δ230−248 along with expression vector for p/CAF-flag in the absence or in the presence of BirA and CA-ALK5 as indicated on top. Western blottings were performed using the anti-flag monoclonal antibody for the detection of p/CAF or horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad proteins.

(−/−) cells strongly increased the transactivation of the G5-E1B promoter by wild type GAL4-Smad3 and its stimulation by TGFβ (Supporting Information Figure 1D). These findings suggested physical and functional interactions between GAL4-Smad3 and Smad4. In contrast, Smad4 failed to increase the transcriptional activity of GAL4-Smad3 Δ230−248 mutant either in the absence or in the presence of TGFβ, suggesting lack of interaction between the two proteins in agreement with the findings of Figure 1D. Similar results were obtained when the Smad3 Δ230−248 mutant was assayed for oligomerization with wild type Smad3. In HepG2 cells, the transcriptional activity of wild type GAL4-Smad3 protein was increased by the simultaneous expression of wild type Smad3 protein indicative of homo-oligomeric interactions between Smad3 molecules (Supporting Information Figure 1E). In contrast, the activity of GAL4-Smad3 could not be increased by the simultaneous expression of the Smad3 Δ230−248 mutant. In fact, the activity was slightly decreased possibly because of a dominant negative or a squelching effect of the mutant versus the wild type Smad3 protein. Similarly, the transcriptional activity of the GAL4-Smad3 Δ230−248 mutant could not be induced by coexpression of wild type Smad3 protein suggesting a lack of interaction between the two proteins (Supporting Information Figure 1E). In control immunoblotting experiments it was shown that the exogenous expression of 6myc-Smad3 or the 6myc-Smad3 (Δ230−248) mutant had no effect on the levels of expression of GAL4-Smad3 or endogenous Smad3 in HepG2 cells (Supporting Information Figure 1F).

The subcellular distribution of wild type Smad3 and the Smad3 Δ230−248 mutant was analyzed by indirect immunofluorescence using 6myc-tagged versions of the two proteins exogenously expressed in COS-7 cells. As shown in Supporting Information Figure 2, the wild type Smad3 displayed a predominant cytoplasmic localization in the absence of the constitutively active ALK5 receptor (top row, -CA-ALK5) whereas in the presence of the coexpressed CA-ALK5, wild type Smad3 accumulated in the nucleus (+CA-ALK5). In contrast, the Smad3 Δ230−248 mutant was localized both in the cytoplasm and in the nucleus in the

absence of CA-ALK5 whereas the overexpression of CA-ALK5 caused an extensive nuclear accumulation of the mutant protein. The data of Supporting Information Figure 2 suggested that the Smad3 Δ230−248 deletion deregulated the TGFβ-induced nucleo-cytoplasmic transport mechanism of Smad3.

Deletion of the 230−248 Region Abolished Smad3 Phosphorylation by the ALK5 Receptor as well as Homo- And Hetero-oligomerization. To investigate further the effect of the 230−248 internal deletion of Smad3 on its homo- and hetero-oligomerization properties, we utilized a protein−protein interaction assay which is based on the biotinylation of one of the interacting proteins *in vivo* by the bacterial biotin ligase BirA (*21*). For this purpose, new expression vectors were constructed bearing the cDNAs of wild type Smad3 or the Smad3 Δ230−248 mutant fused in frame with a small 23 amino acid peptide (Bio) which is a target of the bacterial ligase BirA (Figure 2A). In this system, the simultaneous expression of the Bio-Smad3 fusion proteins and BirA results in Smad3 biotinylation *in vivo* which facilitates the purification of Smad3 and its interacting proteins from cell extracts using streptavidin agarose beads (*21*). Using this system, it was shown that wild type biotinylated Smad3 interacted efficiently with 6myc-Smad2 in a CA-ALK5-dependent manner (Figure 2B, upper blot, lane 3). In contrast, a very weak interaction between 6myc-Smad2 and the biotinylated Smad3 Δ230−248 mutant could be observed which was reduced by CA-ALK5 (Figure 2B upper blot, lanes 5 and 6).

In a similar manner, it was shown that wild type 6myc-tagged Smad3 and 6myc-tagged Smad4 proteins interacted with biotinylated wild type Smad3 in a CA-ALK5-dependent manner (Figure 2C and 2D, upper blot, lane 3). In contrast, no interactions were observed between wild type 6myc-Smad3 or 6myc-Smad4 with the biotinylated Smad3 Δ230−248 mutant (Figure 2C and 2D, upper blot, lane 6). In control experiments, the expression levels of the wild type, 6myc-tagged Smad proteins and the biotinylated Smad3 forms, were monitored by Western blotting using an anti-myc antibody or HRP-conjugated streptavidin (middle and bottom blots of Figure 2B−D).

The lack of ALK5-induced interaction between the Smad3 (Δ230−248) mutant and the other Smads prompted us to investigate the phosphorylation status of this mutant Smad3 protein both in the absence and in the presence of the ALK5 receptor. For this purpose, we utilized an antibody that recognizes Smad3 protein which has been phosphorylated at the C-terminal serine residues 423 and 425 by the ALK5 receptor. As shown in Figure 2D lower blot, the anti-P-Smad3 antibody recognized wild type Smad3 protein which had been phosphorylated by the CA-ALK5 receptor (lane 3) whereas it did not recognize the Smad3 Δ230−248 mutant under the same conditions (lane 6).

We then examined the ability of wild type Smad3 and the Smad3 Δ230−248 mutant to interact physically with the transcriptional coactivator/histone acetyl transferase p/CAF. It had been shown previously that p/CAF interacts physically and functionally with both the linker and the MH2 domains of Smad3 protein (*17*, *25*). As shown in Figure 2E, both wild type Smad3 and the Smad3 Δ230−248 mutant interacted efficiently with p/CAF in a CA-ALK5-dependent manner (upper blot, lanes 4 and 7). As the Smad3 (Δ230−248) mutant cannot be phosphorylated by the ALK5 receptor (Figure 2D), the findings of Figure 2E suggested that ALK5 may induce additional phosphorylations in Smad3 protein that favor interactions with p/CAF (see Discussion).

Molecular Characterization of a Tumorigenic Mutation in Smad4. The 230−248 region in Smad3 is highly conserved among different Smad family members and includes the first and part of the second β strands of the MH2 domain (β1 and β2 strands, Figure 3 shown in yellow). One of these conserved amino acids, glutamic acid 330 in Smad4 has been found to be mutated to alanine in cancer patients (*16*). This amino acid is located in the vicinity of helix H1 (Figure 3 bottom). To functionally characterize this tumorigenic mutation, we introduced the E330A substitution into Smad4 protein by site-directed mutagenesis. We found that this amino acid substitution totally abolished the transcriptional cooperativity between Smad3 and Smad4 on the $(CAGA)_{12}$-E1B promoter in HepG2 cells (Figure 4A). Importantly, we showed that this mutation abolished the ALK5-dependent oligomerization of Smad4 with Smad3 in a protein−protein interaction assay *in vivo* (Figure 4B). Such a defect in oligomerization could account for the inability of Smad4 to activate transcription cooperatively with Smad3 (Figure 4A).

To validate our findings regarding the importance of E330 in Smad oligomerization and transcriptional activation, we introduced the same mutation into the corresponding location of Smad3 protein (E239A). Figure 5 shows that the Smad3 mutant bearing the E239A amino acid substitution (Smad3 E239A) failed to transactivate the $(CAGA)_{12}$-E1B promoter in JEG (Smad3 −/−) cells (Panel A) as well as in MDA-MB-468 (Smad4 −/−) cells in the presence of Smad4 (Panel B). Furthermore, Smad3 E239A could not be phosphorylated by the ALK5 receptor (Panel D, bottom blot, lane 3), and as a consequence, it failed to oligomerize with wild type Smad3 (Panel D, upper blot, lane 3) or wild type Smad 4 (Panel E, upper blot, lane 3) *in vivo* in an ALK5-dependent manner. However, the Smad3 E239A mutant retained its ability to interact physically with the product of the protooncogene c-Ski *in vivo* in agreement with previous reports (Figure 5G, upper blot, lane 6) (*26*−*28*). As a negative control in this experiment, we used a mutant Smad3 protein lacking the MH2 domain (Bio-Smad3 ΔMH2) and showed that this mutant failed to interact physically with c-Ski (Figure 5G, upper blot, lane 9) in agreement with previous findings (*26*−*28*). Immunoblotting analysis showed that the levels of expression of the Smad3 E239A mutant were slightly lower compared to wild type Smad3 (Figure 5C). Similarly to Smad3 (Δ230−248), the Smad3 E239 mutant was constitutively localized in the nucleus when overexpressed in COS-7 cells (Figure 5F).

A Network of Hydrogen Bond and Ionic Interactions Involving Polar Amino Acid Residues in β1 Strand, L2 Loop and H1 α Helix Is Essential for Smad3 Function. According to the previously published crystal structure of a Smad3/Smad4 MH2 heterotrimer [Protein Data Bank accession code (PDB) 1U7F (*29*)], conserved polar amino acid residues of the β1 strand of Smad3 such as E239, participate in a network of hydrogen bond and ionic interactions with other polar amino acids present in helix H1 or the L2 loop that likely contribute to the rigidity of the loop-helix region (*29*). As shown in Figure 6, E239 in Smad3 forms hydrogen bonds with 4 amino acids: Y237 of the β1 strand, N276, R279 of

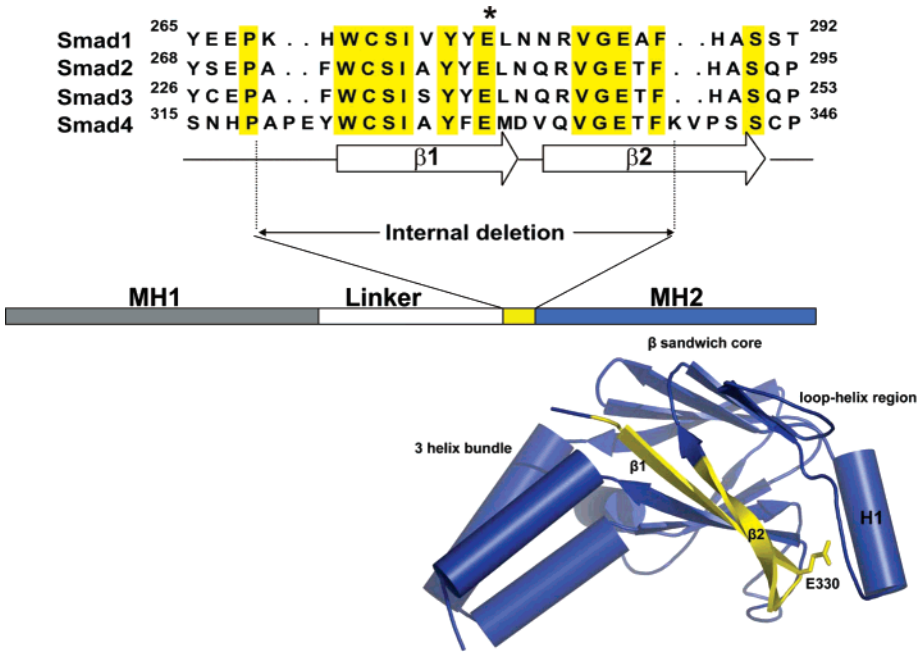

FIGURE 3: Homology between Smad proteins in the 230−248 region of the MH2 domain and MH2 structural features: Amino acid residues which are conserved among TGFβ (Smad2, Smad3) and BMP (Smad1) Smads as well as their common oligomerization partner (Smad4) are shown in yellow. The first two β strands (β1 and β2) of the β sandwich structure of the MH2 domain are shown by thick white arrows. The internal deletion Δ230−248 and the glutamic residue that was mutagenized in Smad3 and Smad4 proteins for the purpose of this study (E330 in Smad4, E239 in Smad3) are shown by the thin arrows and the asterisk respectively. This mutation in Smad4 protein (E330A) is found frequently in cancer patients. The bottom panel shows the structure of the MH2 domain of Smads and the position of all major structural characteristics of this domain (the loop-helix region, the β sandwich core, and the three helical bundle). It also shows the position of the β1 and β2 strands and of amino acid residue E330 in Smad4 in yellow. Note the close proximity of E330 to the α helix H1. The figure was produced with PyMol (http://pymol.sourceforge.net/).

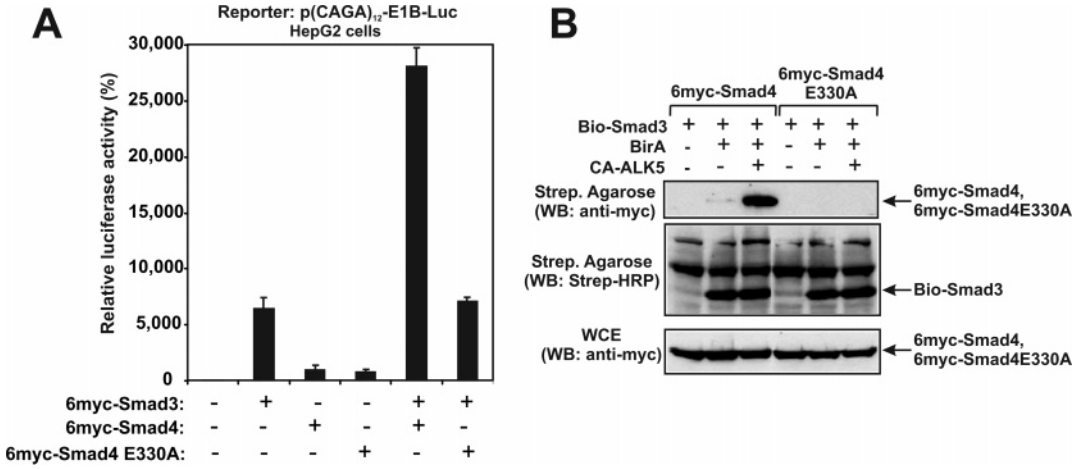

FIGURE 4: A, B: The tumorigenic mutation E330A in Smad4 abolished Smad3/Smad4 oligomerization in response to ALK5 activation. Panel A, Mutation E330A abolished the transcriptional activity of Smad4: Human hepatoma HepG2 cells were transfected with 1 μg of expression vectors for 6myc-Smad3, 6myc-Smad4, or 6myc-Smad4 (E330A) along with the p(CAGA)$_{12}$-E1B-Luc reporter (1 μg). The normalized mean values of luciferase activity ($\pm$SEM) are shown with a histogram. Panel B, Tumorigenic mutation E330A abolished Smad4 oligomerization with Smad3: HEK-293T cells were transfected with expression vectors for Bio-Smad3 and 6myc-Smad4 or 6myc-Smad4 (E330A) in the absence or in the presence of BirA and CA-ALK5 as indicated on top. Western blottings were performed using the anti-myc monoclonal antibody for the detection of myc-tagged Smad4 and Smad4 (E330A) or horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad3 protein. The arrows on the right show the position of the indicated proteins. WCE: whole cell extract. Strep: streptavidin. HRP: horseradish peroxidase.

the L2 loop, and R287 of helix H1. Moreover, the close proximity of a negatively charged amino acid residue (E239) to two positively charged amino acid residues (R279 and R287) is expected to stabilize the structure by neutralizing partly the two positive charges of these two arginine residues. We speculated that mutation E239A in Smad3 or the corresponding E330A mutation in Smad4 described above (Figures 4 and 5) abolished TGFβ signaling and Smad function possibly because of the loss of these hydrogen bond and ionic interactions which could affect the integrity of this region. We hypothesized that mutations in other amino acids participating in this network of interactions could also have a detrimental effect in Smad structure and function. To test our hypothesis, we mutagenized two of these polar amino acids, Y237 and R287, by replacing them with the nonpolar amino acid alanine in order to eliminate the possibility of

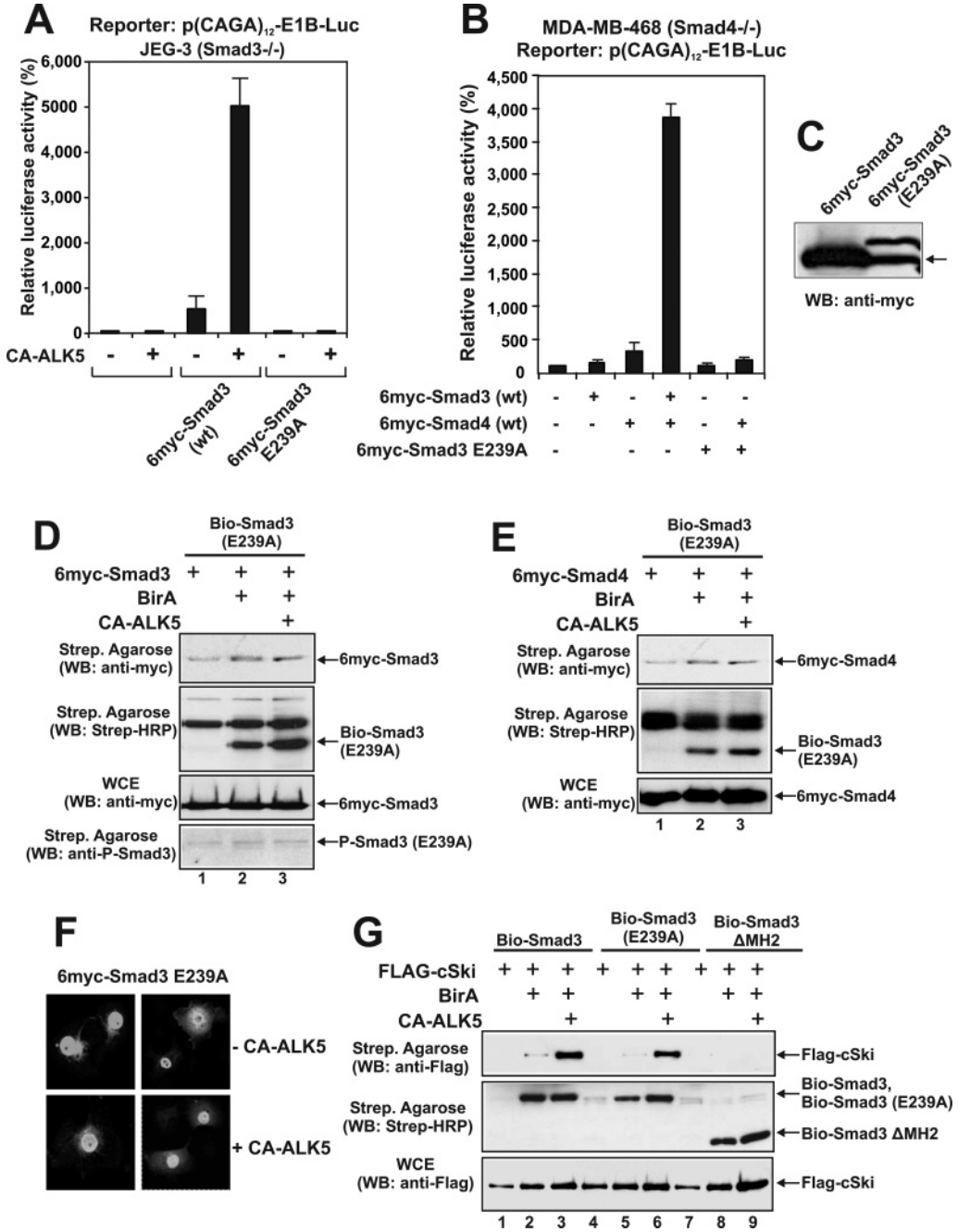

FIGURE 5: A−G: Mutation E239A in Smad3 abolished Smad3/Smad4 oligomerization and transcriptional activation but did not affect interaction of Smad3 with the proto-oncogene c-Ski: Panel A, Human choriocarcinoma JEG-3 cells were transfected with 1 μg of expression vectors for 6myc-Smad3 (wt) or 6myc-Smad3 (E239A) in the absence or in the presence of an expression vector for CA-ALK5 (1 μg) along with the p(CAGA)$_{12}$-E1B-Luc reporter (1 μg). The normalized mean values of luciferase activity (±SEM) are shown with a histogram. Panel B, Breast cancer MDA-MB-468 cells were transfected with the p(CAGA)$_{12}$-E1B-Luc reporter (1 μg) along with expression vectors for 6myc-Smad3 (wt), 6myc-Smad3 (E239A), and 6myc-Smad4 (wt) (1 μg) in the combinations shown at the bottom of the graph. The normalized mean values of luciferase activity (±SEM) are shown with histograms. Panel C, Relative levels of expression of wild type Smad3 and the E239A mutant. HEK293T cells were transfected with equal amounts of expression vectors of 6myc-Smad3 and 6myc-Smad3 (E239A). Equal amounts of whole cell extracts from the transfected cells were subjected to SDS-PAGE and immunoblotting using the anti-myc antibody. Panels D, E, HEK-293T cells were transfected with expression vectors for Bio-Smad3 (E239A) and 6myc-Smad3 (Panel D) or 6myc-Smad4 (Panel E) in the absence or in the presence of BirA and CA-ALK5 as indicated on top. Western blottings were performed using the anti-myc monoclonal antibody for the detection of myc-tagged Smads, horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad3 E239A protein and the anti-phospho Smad3 antibody to detect phosphorylated Smad3 E239A protein (P-Smad3 E239A). The arrows on the right show the position of the indicated proteins. WCE: whole cell extract. Strep: streptavidin. HRP: horseradish peroxidase. Panel F, The Smad3 (Δ230−248) mutant is constitutively localized in the nucleus: COS-7 cells were transfected with an expression vector for the mutant 6myc-Smad3 E239 in the absence (top row) or in the presence (bottom row) of the constitutively active ALK5 receptor. Immunofluorescence was performed as described in Materials and Methods using the anti-myc (9E10) monoclonal antibody followed by a secondary, FITC-conjugated, antibody. Smad proteins were visualized by fluorescence microscopy. Panel G, Mutation E239A does not affect the CA-ALK5-dependent association of Smad3 with the proto-oncogene cSki: HEK-293T cells were transfected with expression vectors for Bio-Smad3, Bio-Smad3 (E239A) or Bio-Smad3 (MH2) along with an expression vector for FLAG-cSki in the absence or in the presence of BirA and CA-ALK5 as indicated on top. Western blottings were performed using the anti-flag monoclonal antibody for the detection of cSki or horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad proteins.

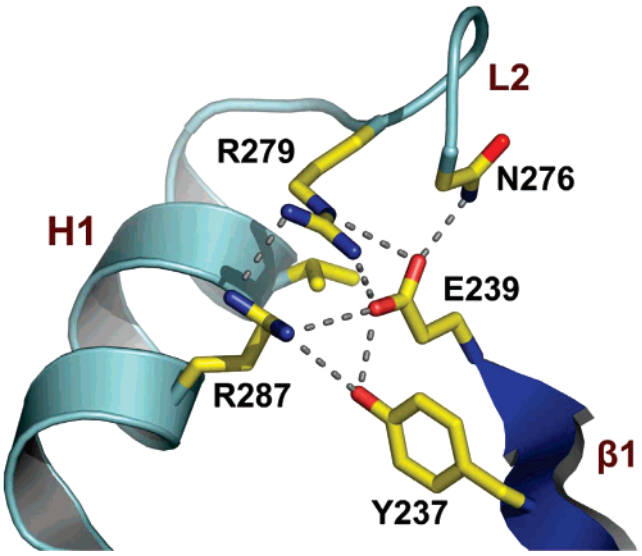

FIGURE 6: Structure of the Smad3 MH2 domain in the region surrounding glutamic acid residue 239. The structure reveals a network of hydrogen bonding interactions between E239 and several amino acid residues that are located either in β1 (Y237), L2 (N276, R279), and H1 (R287). The hydrogen bonds are shown as dashed lines. Note that ionic interactions between positively charged (R279, R287) and negatively charged (E239) amino acids could also contribute to the stability of the structure. The figure was produced with PyMol (http://pymol.sourceforge.net/).

hydrogen bond or ionic interactions with neighboring residues and examined the new Smad3 mutants for oligomerization and transcriptional activation. As shown in Figure 7, mutation R287A totally abolished the ability of Smad3 to oligomerize with Smad4 and with Smad3 in an ALK5-dependent manner (Panels A and B, upper blot lane 9). In contrast, mutation Y237A behaved as wild type Smad3 as it interacted effectively with both Smad3 and Smad4 (Panels A and B, upper blot lane 6). Immunoblotting analysis using an antibody that recognizes phosphorylated Smad3 showed that Smad3 R287A was phosphorylated by the ALK5 receptor albeit less effectively than wild type Smad3 or the Y237A mutant (Figure 7A, bottom blot, compare lanes 3 and 6 with lane 9). Finally, we showed that the R287A mutation abolished transactivation of the $(CAGA)_{12}$-E1B promoter in MDA-MB-468 cells in the presence of Smad4 whereas the Y237A mutation behaved as wild type Smad3 and transactivated strongly the $(CAGA)_{12}$-E1B promoter in the presence of Smad4 and the CA-ALK5 receptor (Figure 7C).

DISCUSSION

In an effort to identify and characterize novel domains in Smad proteins which play essential roles in their functions as TGFβ signaling mediators, we initially focused our attention to a short region at the junction between the linker and the MH2 domain of Smad3 (aa 230−248). The focus on this region came as a result of our previous structure−function analysis of human Smad3 protein which had indicated that this region is required for the transcriptional activity of both the linker and the MH2 domains (*17*). This region is rich in conserved polar residues and includes the first and part of the second β strands of the MH2 domain (Figure 3).

Importantly, a single amino acid substitution within the β1 strand of Smad4 (E330A) was found to be frequently associated with tumors (*16*). In a previous study, it was shown that the colon cancer cell line VACO-235 expresses a Smad4 protein bearing a double amino acid substitution (E330N/D355A) (*30*). In these cells, the classical TGFβ/Smad pathway was not operating, but the cells were responsive to the growth inhibitory effects of TGFβ. The authors suggested that growth inhibition in these cells is mediated by an alternative non-Smad signaling pathway. However, this double Smad4 mutant was not characterized further in that report.

In the present study, we showed that a Smad4 mutant bearing a single point mutation at position E330, (Smad4 E330A) was transcriptionally inactive in a classic SBE-Luc transactivation assay (Figure 4A), and furthermore we showed that the same mutation abolished the TGFβ-dependent oligomerization of Smad4 with Smad3 (Figure 4B). We proceeded further into the characterization of this region and this residue in particular by showing that the corresponding mutation in Smad3 (E239A) severely affected the ALK5-dependent phosphorylation, oligomerization, and transcriptional activation (Figure 5). A comparison of the data from the analysis of the two mutants (Smad3 E239A and Smad4 E330A) presented in Figures 4 and 5 indicated that the defect in Smad oligomerization caused by this mutation could not be accounted for only by a defect in Smad phosphorylation by the ALK5 receptor as Smad4 cannot be phosphorylated by this receptor (*7*). Thus, mutagenesis of this specific glutamic acid residue in the β1 strand of R-Smads or Smad4 may directly affect the physical interactions between MH2 subunits.

Of interest was the observation that, in contrast to other previously characterized tumorigenic mutations in Smads that map at the interfaces between interacting MH2 subunits, E239 does not map at these interphases. In the previously published crystal structure of the Smad3/Smad4 MH2 heterotrimer [Protein Data Bank accession code, 1U7F (*29*)], E239 is facing toward the adjacent H1 helix and participates in a dense network of hydrogen bond and ionic interactions with other amino acid residues that are located in the β1 strand, the L2 loop, or the helix H1 (Figure 6). As shown in Figure 6, the carboxyl group of the side chain of glutamic acid 239 could serve as a hydrogen donor or acceptor in hydrogen bonds with four different amino acids: N276 and R279 of L2, Y237 of β1, and R287 of H1. Furthermore, the same carboxyl group of E239 is located in close proximity with the two positively charged side chains of arginines 279 and 287, and this ionic interaction is expected to stabilize the structure by partly neutralizing the two positive charges. Thus, the replacement of the polar side chain of glutamate with a nonpolar one (alanine) is predicted to disrupt all these hydrogen and ionic bonds.

The highly conserved residue tyrosine 237 (Figure 3) was expected to participate actively in this network because of the polar character of the hydroxyl group of tyrosine, which hydrogen bonds with both E239 and R287 (Figure 6) and because the phenol group could also be involved in hydrophobic interactions with other nonpolar residues in the vicinity thus contributing to the rigidity of this structure. Unexpectedly however, we found that substitution of tyrosine 237 to alanine (mutant Y237A) had no phenotype in all

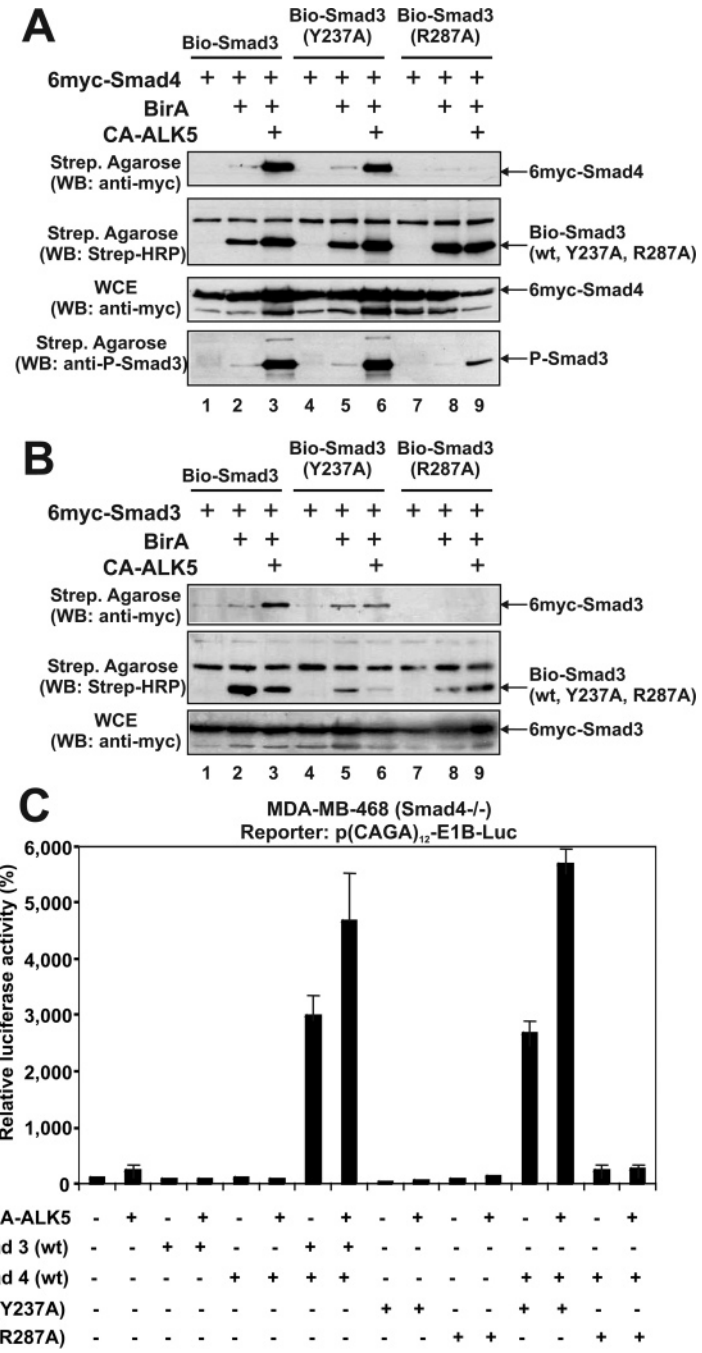

FIGURE 7: A−C: Effect of Y237A and R287A mutations in the oligomerization and transactivation properties of Smad3 protein. Panels A and B, HEK-293T cells were transfected with expression vectors for Bio-Smad3, Bio-Smad3 (Y237A), and Bio-Smad3 (R287A) along with 6myc-Smad4 (Panel A) or 6myc-Smad3 (Panel B) in the absence or in the presence of BirA and CA-ALK5 as indicated on top of each Panel. Western blottings were performed using the anti-myc monoclonal antibody for the detection of myc-tagged Smad3 and Smad4 proteins, an anti-P-Smad3 antibody for the detection of phosphorylated Smade proteins or horseradish peroxidase-conjugated streptavidin for the detection of biotinylated Smad3 proteins. WCE: whole cell extract. Strep: streptavidin. HRP: horseradish peroxidase. Panel C, Breast cancer MDA-MB-468 cells were transfected with the $p(CAGA)_{12}$-E1B-Luc reporter (1 μg) along with expression vectors for 6myc-Smad3, 6myc-Smad3 (Y237A), 6myc-Smad3 (R287A), and 6myc-Smad4 (1 μg) in the absence or in the presence of CA-ALK5 (1 μg) in the combinations shown at the bottom of the graph. The normalized mean values of luciferase activity (±SEM) are shown with the histogram.

functional assays (oligomerization, receptor phosphorylation, and transcriptional activation) suggesting that the polar character of this residue's side chain may not be crucial for its contribution to MH2 structure, and some of the hydrogen bonds that are indicated by the crystal structure may not actually occur. Thus, this mutation in Smad3 is another example of the significance of the structure−function analysis in the elucidation of the role of individual amino acid residues in a protein's function.

On the other hand, the substitution of arginine 287 to alanine had dramatic consequences in Smad3 function such as the homo- and hetero-oligomerization and the transcriptional activation (Figure 7). Similar to the E239A and Y237A mutations, this substitution was designed in order to eliminate the ability of this amino acid residue to form hydrogen or ionic bonds with neighboring amino acids such as Y237, E239, and R279 which were indicated from the crystal structure (Figure 6). In addition to these intramolecular

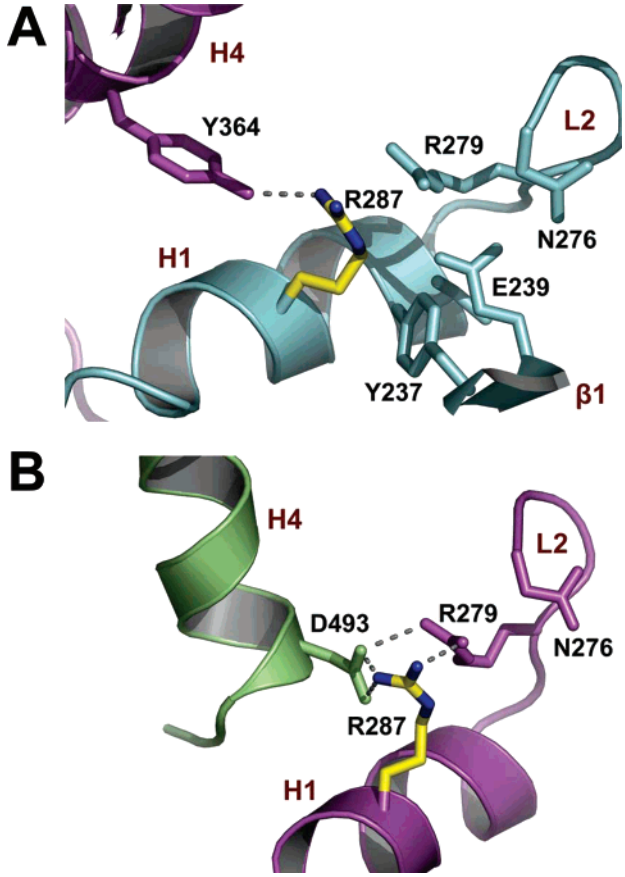

FIGURE 8: A, B: Structure of the Smad3/Smad4 MH2 heterotrimer (PDB: 1U7F) in the region surrounding arginine 287 in Smad3. Panel A: Smad3−Smad3 intermolecular interactions facilitated by R287 in MH2 subunit A (cyan) and Y364 in MH2 subunit C (magenta). The hydrogen bonds are shown as dashed lines. Panel B: Smad3−Smad4 intermolecular interactions facilitated by R287 in Smad3 (MH2 subunit A in magenta) and D493 of Smad4 (MH2 subunit B in lime). The hydrogen bonds are shown as dashed lines.

interactions which seem to be essential for the rigidity of the MH2 domain, the crystal structure of the Smad3/Smad4 MH2 hetero-trimer [PDB 1U7F] shows that R287 is also involved in intermolecular interactions with adjacent MH2 subunits. More specifically, it interacts via hydrogen or ionic bonds with Y364 of the second Smad3 MH2 subunit and with D493 of the Smad4 MH2 subunit in the heterotrimer (Figure 8A and B respectively), and the loss of these interactions by the side chain substitution may easily account for the oligomerization defect. Of notice was also the observation that in contrast to the E239A mutation which could not be phosphorylated by the ALK5 receptor, in the case of the R287A mutation, the phosphorylation by the ALK5 receptor still occurs (Figure 7A), suggesting that the oligomerization defect in the latter mutant was the result of the loss of direct contacts between Smad subunits following the phosphorylation by the TGFβ receptor type I.

As discussed above, the defect in Smad oligomerization caused by the E239A mutation could not be accounted for only by a defect in Smad phosphorylation by the ALK5 receptor, as Smad4 cannot be phosphorylated by the type I TGFβ receptor (*7*). However, the loss of phosphorylation of the Smad3 E239A mutant by the ALK5 receptor is of special interest. Smad oligomerization, which is triggered by the phosphorylation of R-Smads by the type I TGFβ receptor following ligand stimulation, is at the core of TGFβ signaling (*31*). Previous studies have identified the L3 loop of the MH2 domain of Smad proteins as essential for their interaction with the corresponding type I receptor and for conferring signaling specificity (*32*). Mutations in the L3 loop abolished Smad activation by the TGFβ type I receptor (*32*). On the basis of the above, we are tempted to propose that the E239A mutation as well as the more drastic Δ230−248 internal deletion may have caused similar conformational changes in Smad3 protein that prevented its physical association with the ALK5 receptor. Thus, our findings suggest that in addition to the L3 loop, elements of the loop-helix region of Smads may also contribute to Smad activation by the type I TGFβ receptor and to their ability to mediate TGFβ-induced responses including oligomerization and transcriptional activation.

Another interesting observation that came from this study was that the phosphorylation-deficient Smad3 mutants Δ230−248 and E239A were still able to interact physically with the histone acetyltransferase p/CAF and the product of the c-ski proto-oncogene respectively *in vivo*, in an ALK5-dependent manner (Figures 2E and 5G). In the case of Smad3-p/CAF interaction, given that p/CAF interacts with two different domains in Smad3, the MH2 domain and the linker region (*17*, *25*), we suggest that the enhanced interaction with p/CAF (and possibly with c-Ski) is due to Smad3 phosphorylation at additional sites, possibly in the linker region. This is in good agreement with recent data showing that TGFβ enhances the linker phosphorylation of a Smad3 mutant that cannot be phosphorylated at the C-terminus (*33*). The utilization of the recently identified Smad linker phosphatases SCP1−3 (*33*) should help clarify the role of linker phosphorylation in several Smad3 functions including their physical interaction with coregulatory factors.

Alignment of the amino acid sequences of Smad proteins from different species showed that the region of Smad3 between amino acids 230 and 290 is evolutionarily conserved (Supporting Information Figure 3). Certain amino acids in this region including the residues analyzed in this study (Y237, E239, N276, R279 and R287) are identical in Smad3 from all species examined as well as in bone morphogenetic protein (BMP)-regulated Smads 1 and 5 and in the common partner Smad4, suggesting a crucial role for these residues in Smad structure and function and an evolutionary conserved mechanism of Smad oligomerization.

Finally, as discussed above, based on the published crystal structure of the Smad3/Smad4 MH2 heterotrimer, the R287A mutation is predicted to abolish the interaction between R287 in Smad3 and D493 in Smad4 (Figure 8B). A mutation in residue D493 of Smad4 has been identified frequently in cancer patients (*34*). More specifically, in these patients, aspartic acid 493 has been mutagenized to histidine (D493H). Given the different polar characters of histidine and aspartate (the imidazole ring of histidine side chain is almost uncharged at neutral pH in contrast to the carboxyl group of aspartate which is negatively charged), we predict that histidine at position 493 in Smad4 could not be able to make hydrogen bonds or ionic interactions with arginines 287 and 279 (Figure 8B) but should move to a different position in order to avoid steric clashes (distances <2 Å) with these amino acids. Thus, the tumorigenic mutation D493H in Smad4 could be explained in part by loss of intermolecular interactions with Smad3 at position R287.

In conclusion, our mutagenesis analysis presented here revealed for the first time the relative contribution of individual polar amino acids to the structure and function of the MH2 domain of Smad proteins and also provides a molecular basis for previously uncharacterized tumorigenic mutations in Smad proteins.

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SUPPORTING INFORMATION AVAILABLE

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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