30 min, the cells were washed 5 times with 200 μ l PBS + 0.1% TritonX-100, resuspended in 6 μ l mounting media and placed on a slide. The sensitivities of wild-type, *cof1-5* and *cof1-22* cells to lat-A were examined in a halo-assay¹¹ in which filters spotted with Lat-A were deposited on lawns of yeast and the diameter of zones of growth inhibition measured. For actin-patch movement studies, the *COF1* and *cof1-22* strains were transformed with a plasmid carrying GFP linked to Abp1 (ref. 19). Cells from such transformants were grown at 20 °C to an A_{600} of ~0.4, and movement of actin patches was followed with Zeiss Axiovert 100TV microscope. Images from about 400 patches in 5 unbudded cells from each strain were collected using a Princeton Instruments ST-138 camera/controller with a Kodak KAF1400 chip (each pixel is 0.069 μ m $\times$ 0.069 μ m). Frames from the cells were recorded at intervals of 0.95 to 1.25 s and the data analysed using the Animate program (written by A. Mallavarapu). The maximum speeds observed in our study (0.75 μ m s⁻¹) are approximately twofold slower than the maximum speeds previously reported¹⁸. This might result from differences in yeast strain backgrounds or the differences in the time resolution of image acquisition (the camera system in ref. 18 recorded frames with 0.2-s intervals compared to ~1-s intervals in this study).

In vitro depolymerization assay. The *COF1* yeast cofilin gene was amplified by PCR, subcloned into pGEX2T vector (Pharmacia), and expressed in *Escherichia coli* JM109 cells as a glutathione S-transferase (GST) fusion protein. Site-directed mutations were introduced to pGEX2T-*COF1* plasmid by oligonucleotide-based mutagenesis (Transformer, Clontech). Fusion proteins were purified by glutathione-agarose affinity chromatography²⁹. All constructs made by PCR were sequenced to exclude the possibility of undesired mutations. Cofilin was cleaved from GST by thrombin and purified by gel-filtration (Superdex-75, Pharmacia). Yeast actin was purified as described³⁰ and polymerized in F-buffer comprising (in mM) 20 Tris, pH 7.5, 100 KCl, 2 MgCl₂, 0.7 ATP, 0.2 DTT, 0.2 CaCl₂, at a final concentration of 5 μ M. For the depolymerization assay, F-actin and cofilin were mixed in a cuvette to final concentrations of 0.5 μ M, and the depolymerization of F-actin was followed by decrease in light scattering at 400 nm.

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Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4

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Smad2 and Smad4 are related tumour-suppressor proteins^{1,2}, which, when stimulated by the growth factor TGF- β , form a complex to inhibit growth³. The effector function of Smad2 and Smad4 is located in the conserved carboxy-terminal domain (C domain) of these proteins and is inhibited by the presence of their amino-terminal domains (N domain)^{4,5}. This inhibitory function of the N domain is shown here to involve an interaction with the C domain that prevents the association of Smad2 with Smad4. This inhibitory function is increased in tumour-derived forms of Smad2 and 4 that carry a missense mutation in a conserved N domain arginine residue. The mutant N domains have an increased affinity for their respective C domains, inhibit the Smad2–Smad4 interaction, and prevent TGF β -induced Smad2–Smad4 association and signalling. Whereas mutations in the C domain disrupt the effector function of the Smad proteins, N-domain arginine mutations inhibit SMAD signalling through a gain of autoinhibitory function. Gain of autoinhibitory function is a new mechanism for inactivating tumour suppressors.

SMAD proteins are central to signalling by the TGF- β family of growth factors⁶. An effector function of the C domain and an inhibitory function of the N domain of Smad proteins were discovered during studies on the mesoderm-inducing activity of Smad2 in *Xenopus* embryo explants⁵ and on the transcriptional activity of various Smad proteins in mammalian cells⁴. SMAD proteins exist as homo-oligomers that form functional hetero-oligomeric complexes in response to TGF- β and related factors³. These complexes are formed by association of a pathway-restricted SMAD with the shared SMAD, Smad4 (also known as DPC4 or deleted in pancreatic carcinoma 4). Thus Smad1 associates with Smad4 in response to stimulation by bone morphogenic protein

To investigate the domains involved in these interactions, we tested various Smad4 fragments as baits either against Smad4, to detect homo-oligomeric interaction, or against Smad2, to detect hetero-oligomeric interaction, in a yeast two-hybrid system. Full-length Smad4 interacted with the N domain/linker region as a whole but not with those two regions when they were separately expressed (Fig. 1a). Full-length Smad4 interacted with its isolated C domain (Fig. 1a). These results indicate that both the C domain and the N domain/linker region can contribute to the homo-oligomeric interaction in Smad4 (Fig. 1a). Furthermore, the isolated C domain of Smad4 interacted with itself (data not shown), which was unexpected¹¹. The homo-oligomeric interaction pattern of

The Smad2–Smad4 complex was detectable in yeast and was particularly sensitive to deletions in the C domain (Fig. 1a). Furthermore, Smad2 and Smad4 (Fig. 1a) or their isolated C domains (data not shown) interacted with the other's isolated C domain. In contrast to its dependence on TGF- β stimulation in mammalian cells³, the Smad2–Smad4 interaction occurred spontaneously in yeast, perhaps as a result of Smad phosphorylation by yeast kinases. These interactions were also analysed in mammalian cells by transfection of Smad2 and Smad4 fragments tagged with a Flag or HA epitope. The isolated C domains of Smad2 and 4 each

248–467). Versions of the same protein tagged at the amino terminus with the Flag epitope or at the carboxy terminus with the HA epitope were co-transfected. Some cultures were incubated with TGF- β for 1 h before lysis. Homooligomerization was analysed by anti-HA immunoblotting of anti-Flag immunoprecipitates. **d**, Hetero-oligomerization of cotransfected HA-tagged Smad4 and Flag-tagged Smad2 deletion constructs. Smad2–Smad4 interaction was analysed by anti-Flag immunoblotting of anti-HA immunoprecipitates (top) or anti-HA immunoblotting of anti-Flag immunoprecipitates (bottom). **e**, Summary of SMAD domain contributions to Smad2–Smad4 hetero-oligomerization.

formed homo-oligomers in COS cells, as determined by co-immunoprecipitation of differently tagged constructs (Fig. 1c). In agreement with previous reports³, the Smad2–Smad4 interaction in COS cells requires TGF- β receptor stimulation (Fig. 1d), whereas the homo-oligomeric interactions do not (Fig. 1c). The C domains of Smad2 and Smad4 were necessary and sufficient for this interaction (Fig. 1d).

Deletion of the N domain in either Smad2 or Smad4 caused a constitutive association with the full-length version of the other protein, and this association was further enhanced by TGF- β action (Fig. 1d). The amount of expression of the Smad2 and Smad4 deletion products was similar to that of the full-length proteins (Fig. 1b), arguing that the constitutive association was not due to higher

expression of the N domain-deletion products. Thus, the combined results in yeast and COS cells indicate that the Smad2–Smad4 interaction is mediated primarily by the C domains, requires the integrity of these domains, and is inhibited by the presence of the N domains (Fig. 1e).

Consistent with this inhibitory function, expression of the Smad4 N domain (Fig. 2a) or the Smad2 N domain (Fig. 2b) inhibited the association between full-length as well as the C-domain forms of Smad2 and Smad4 in COS cells. This effect is specific because the overexpression of N domains does not inhibit homo-oligomerization of the C domains (Fig. 2c) or the expression of cotransfected C domains (data not shown). In yeast, the isolated N domains of Smad2 and 4 interacted weakly with their respective C domains (data not shown) and not at all with the full-length proteins (Fig. 1a). However, a fusion protein of glutathione-S-transferase (GST) with the Smad2 N domain specifically bound the Smad2 C domain when tested as recombinant proteins *in vitro* (Fig. 2d). Furthermore, the Smad2 and 4 N domains interacted with their corresponding C domains in COS cells (Fig. 5c). The more efficient interaction of the Smad N and C domains in COS cells may require a post-translational modification or an accessory factor.

As the formation of Smad2–Smad4 complexes requires Smad2 phosphorylation¹⁰ at C-terminal serines^{9,10}, we investigated how this

Figure 2 **a, b**, Inhibition of Smad2–Smad4 interaction by N domains. Increasing amounts (1, 2 and 4 μ g) of plasmid encoding the Smad4 N domain (amino acids 1–154) or the Smad2 N domain (amino acids 1–185) were co-transfected with the indicated full-length or C domain forms of Smad4 and Smad2. Smad2–Smad4 association was then determined. N domain and Smad4 expression was monitored by immunoblotting. **c**, N domain expression does not affect C domain homo-oligomerization. Flag-tagged and HA-tagged versions of Smad C domains were co-transfected with the indicated N domain. **d**, Specific interaction of the Smad2 N domain with the Smad2 C domain *in vitro*. Bacterially expressed proteins (Coomassie-blue-stained gel, left) were used to demonstrate Smad2(C) binding to GST–Smad2(N) but not to GST alone (right).

Figure 3 Effect of N domain deletion and agonist-induced phosphorylation on Smad2–Smad4 interaction. **a**, Constitutive association of the isolated C domains of Smad4 and Smad2, and further stimulation by TGF- β . The indicated full-length or C domain forms of Smad2 or Smad4 were cotransfected into COS cells. Cultures were stimulated with TGF- β , and Smad2–Smad4 interactions were analysed. **b**, Smad2 C domain phosphorylation in response to TGF- β . Constructs were transiently co-transfected with T β R-I into R-1B/L17 cells. Transfectants were labelled with ³²P-orthophosphate, stimulated with TGF- β for 20 min, and immunoprecipitated with anti-Flag antibody. **c**, The constitutive interaction of Smad4 and Smad2 C domains is independent of TGF- β -receptor-mediated phosphorylation. Smad2–Smad4 (full-length or C domain) complex formation was analysed in the presence or absence of a cotransfected dominant negative T β R-I construct (T β R-I(KR)).

process related to the inhibitory effect of the N domain. The Smad2 and Smad4 C domains spontaneously associated with each other and this association was further stimulated by TGF- β (Fig. 3a). The phosphorylation of the Smad2 C domain in response to TGF- β was comparable to that of full-length Smad2 (Fig. 3b). Co-transfection of a kinase-inactive, dominant-negative TGF- β type-I receptor [T β R-I(K232R)]¹³ abolished the TGF- β -stimulated association of Smad2 and Smad4 C domains but not their constitutive association (Fig. 3c). Thus the Smad2–Smad4 interaction is stimulated not only by phosphorylation of the Smad2 C domain¹⁰ but also by removal of the N domains.

We next investigated Smad2 and Smad4 products containing N-domain mutations that had been identified in human cancers^{2,14}. Smad4 mutations have been found in cancers of the pancreas, colon, oesophagus, breast, ovary, and head and neck^{1,14–17}; Smad2 mutations have been found in colon and head and neck cancers^{2,18,19}. Most of the missense mutations reported so far are in the C domains of Smad2 or 4. The three-dimensional structure of the Smad4 C domain predicts that some of these mutations destabilize the core

structure, others disrupt the C-domain homotrimer interface, and others disrupt a putative Smad4–Smad2 interface¹². Missense mutations have also been identified in the N domains of Smad2 and Smad4, including a Smad2 Arg133Cys mutation in a colon carcinoma² and a Smad4 Arg100Thr mutation in a pancreatic carcinoma¹⁴. Arg 133 in Smad2 corresponds to Arg100 in Smad4 (ref. 2), both of which are located in a highly conserved region of the SMAD proteins, suggesting that mutations at this residue are selected in cancer.

We confirmed that these N-domain mutations inactivate the signalling function of Smad2 and Smad4. In the *Xenopus* embryo, injection of *Smad2* transcripts mimics the ability of activin to induce dorsal mesoderm in ectodermal explants^{5,20}. The Arg133Cys mutation prevented *Smad2* from inducing muscle actin, a paraxial mesoderm marker, in this assay (Fig. 4a). In the human breast carcinoma cell line MDA-MB468, which has a *Smad4* homozygous deletion¹⁴ and is therefore insensitive to TGF- β (ref. 3), *Smad4* transfection restores TGF- β sensitivity; this sensitivity is enhanced by co-transfection of *Smad2* (ref. 3). Using the TGF- β reporter gene construct 3TP-luciferase¹³, we found that mutation of Arg 133 in Smad2 or Arg100 in Smad4 stopped the co-transfected constructs from restoring TGF- β responsiveness in these cells (Fig. 4b). Moreover, Smad4 overexpression inhibits the proliferation of MDA-MB468 cells³, which was also disrupted by the Arg100Thr mutation (Fig. 4c).

To determine the basis for the inactivating effect of these N domain mutations, we transfected mutant Smad2 and Smad4 into COS cells and investigated their expression and interactions. Expression of both mutants was similar to wild type (Fig. 5a), as was their ability to form homo-oligomers (Fig. 5b). But both mutants failed to form Smad2–Smad4 complexes in response to TGF- β (Fig. 5b). As the Smad2–Smad4 interaction is primarily a function of the C domains and is repressed by the N domains, we investigated the inhibitory function of the wild-type and mutant N domains.

When expressed as separate polypeptides in COS cells, the N domains of Smad2 or Smad4 associate with the corresponding C domain (Fig. 5c). This association is not observed between the N domain of one SMAD and the C domain of the other (Fig. 5c). The Smad2 and Smad4 mutant N domains interacted with the corresponding C domains more strongly (18- and 22-fold, respectively) than did the wild-type N domains (Fig. 5c). As these mutations do not increase expression of the N domains, this increase in binding probably results from an increase in affinity of the mutant N domains for the C domains. Furthermore, the mutant N domains were more effective than wild-type N domains at inhibiting Smad2–Smad4 hetero-oligomerization (Fig. 5d).

To determine the effect of the Smad4 N domain on signalling by Smad2–Smad4, we tested its effect on the activation by Smad2 and Smad4 of the 3TP-luciferase reporter. In agreement with previous studies³, overexpression of Smad2 and Smad4 or of their C domains activated this reporter (Fig. 5e). Co-transfection of the Smad2 or Smad4 N domains significantly inhibited this effect (Fig. 5e). Furthermore, the mutant N domains were more potent inhibitors than the wild-type N domains of the Smad2/Smad4-mediated response (Fig. 5e). Thus, the binding of a Smad N domain to the corresponding C domain correlates with inhibition of the Smad2–Smad4 interaction and signalling function. We conclude that the Arg133Cys and Arg100Thr mutations increase the inhibitory function of the Smad2 and Smad4 N domains and inactivate signalling by Smad2–Smad4. As Smad4 partners other SMAD proteins besides Smad2 (refs 3, 7), the Arg100Thr mutation should disrupt signalling by these proteins as well.

We have shown that the N domain in SMAD proteins interacts with and represses the effector function of the C domain, and that certain Smad2 and Smad4 mutations found in human cancer cells inactivate these proteins by augmenting the inhibitory function of

Figure 4 Biological activity of Smad2 and Smad4 containing tumour derived N-domain mutations. **a**, Wild-type Smad2 induces the paraxial mesoderm marker muscle actin in *Xenopus* ectodermal explants, whereas Smad2(R133C) or its N domain alone (Smad2(N/R133C)) are unable to induce it. EF-1 α was used as an internal control. **b**, Cotransfection of wild-type Smad2 and Smad4 (WT) restores TGF- β responsiveness into Smad4-defective MDA-MB468 breast cancer cells, whereas cotransfections including the Smad2(R133C) mutant (R), the Smad4(R100T) mutant (R), or both mutants, do not. The TGF- β responsiveness of these cells was determined using the reporter construct 3TP-lux. **c**, Overexpression of wild-type Smad4 inhibits MDA-MB468 cell proliferation, whereas overexpression of the Smad4(R100T) mutant does not. The proliferation of the cells was determined by measuring iododeoxyuridine incorporation into DNA. Results are the average $\pm$ s.d. of triplicate assays.

Figure 5 Gain of autoinhibitory function of Smad4 and Smad2 N domain mutants. **a**, Expression of wild-type and mutant Smads were determined by epitope-tag immunoprecipitation from 35 S-methionine-labelled transfected COS cells. **b**, N domain mutations inhibit Smad2-Smad4 interaction. HA-tagged wild-type or mutant Smad4 was co-transfected with Flag-tagged Smad4 (for homo-oligomeric interaction) or Flag-tagged Smad2 (for hetero-oligomeric interaction) in COS cells. Likewise, Flag-tagged wild-type or mutant (R133C) Smad2 was cotransfected with HA-tagged Smad2 or HA-tagged Smad4. **c**, N domain interaction with the C domain, and its enhancement by mutation. Flag-tagged N domains were cotransfected with the HA-tagged C domains indicated at the bottom. N domain interaction with the C domain was determined by anti-HA immunoblotting of anti-Flag immunoprecipitates. N domain expression was monitored by immunoprecipitation from 35 S-methionine-labelled cells. **d**, Mutant N domains

inhibit the Smad2-Smad4 interaction more strongly. Increasing amounts of plasmid DNA encoding wild-type or mutant (R100T) Smad4 N domain (left) or wild-type or mutant (R133C) Smad2 N domain (right) were cotransfected with Flag-tagged Smad2 C domain and HA-tagged Smad4 C domain. The amounts of Smad4 or Smad2 N domain protein and Smad2(C)-bound Smad4(C) were determined by immunoblotting, quantified (ImageQuant; Molecular Dynamics) and plotted against each other. **e**, N domain inhibition of Smad2-Smad4 signalling. R-1B/L17 cells were transiently transfected with the indicated constructs and 3TP-lux reporter. Amounts of transfected Smad4 and Smad2 were adjusted³ to increase luciferase expression synergistically. Increasing amounts (1, 2, 4 and 6 μ g) of plasmid DNA encoding wild-type or mutant N domains were cotransfected with a Smad4(C)/Smad2(C) combination. Results (luciferase activity in arbitrary units) are the average $\pm$ s.d. of triplicate assays.

the N domain. Other tumour-suppressor mutations, including missense mutations in the C domains of Smad2 and Smad4, act by disrupting protein stability or effector function¹². Our findings reveal a mechanism of tumour suppressor inactivation which instead involves a gain of autoinhibitory function. Antagonists of SMAD autoinhibition might be useful in reversing the effects of this type of mutation. □

Methods

Construction of expression vectors. To generate human Smad4 and Smad2 mutations, a fragment of the corresponding cDNAs^{1,4} was amplified by PCR. The amplified region was subcloned into full-length Smad4 or Smad2 in pCMV5 for transfection into mammalian cells. The regions amplified by PCR and the presence of missense mutations were confirmed by sequencing.

Yeast two-hybrid system. LexA fusions were created in pBTM 116 (ref. 21) and GAD fusions within pGAD424 (Clontech). Interactions were tested in the strain L40 (ref. 22). Activation of the LexA operator–HIS3 reporter was assayed on medium lacking histidine with increasing concentrations of 3-amino-triazole.

Transfection, immunoprecipitation, immunoblot and metabolic labelling. For Smad2/Smad4 homo- or hetero-complex analysis, COS cells were transiently transfected with the indicated constructs and stimulated with 200 pM TGF- β 1 for 1 h. Cells were lysed in TNE buffer³, immunoprecipitated with anti-Flag M2 monoclonal antibody (IBI; Eastman Kodak), and interacting proteins detected by immunoblotting with the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) as described³. Anti-SMAD rabbit polyclonal antibody was raised against full-length Smad1. To study the interaction between the N and C domains of Smad4 or Smad2, transiently transfected COS cells were lysed in LSLD buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% Tween 20, 10% glycerol, 1 mM DTT) containing protease and phosphatase inhibitors. Immunoprecipitation and immunoblotting were done as described. COS or R-1B/L17 cells transfected with the indicated constructs were labelled with ³⁵S-methionine or ³²P-orthophosphate and visualized by electrophoresis and autoradiography²³.

In vitro binding. The Smad2 N domain (amino acids 1–184) fused to GST and the Smad2 C domain (amino acids 248–467) protein were expressed in *Escherichia coli* and partially purified by column chromatography. GST fusion proteins were conjugated to glutathione–Sepharose beads (Pharmacia) and incubated with Smad2 C domain protein. Smad2 C domain protein was detected by western blotting using anti-Smad antibody.

Functional assays. For the animal cap assay, RNA (10 nl, 2 ng) was introduced in the animal pole of two-cell *Xenopus* embryos. Animal caps were explanted at the blastula stage and cultured to the tadpole stage. Total RNA from the explants and control sibling embryos was extracted and was amplified with reverse transcription by using muscle actin and *EF-1 α* primers²⁴. In the MDA-MB468 cell experiments, the amount of transfected plasmids was adjusted to render the TGF- β response dependent on both Smad2 and Smad4. Luciferase and growth inhibition were assayed as described³.

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A structural basis for mutational inactivation of the tumour suppressor Smad4

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The Smad4/DPC4 tumour suppressor¹ is inactivated in nearly half of pancreatic carcinomas² and to a lesser extent in a variety of other cancers^{2–4}. Smad4/DPC4, and the related tumour suppressor Smad2, belong to the SMAD family of proteins that mediate signalling by the TGF- β /activin/BMP-2/4 cytokine superfamily from receptor Ser/Thr protein kinases at the cell surface to the nucleus^{5–7}. SMAD proteins, which are phosphorylated by the activated receptor, propagate the signal, in part, through homo- and hetero-oligomeric interactions^{8–13}. Smad4/DPC4 plays a central role as it is the shared hetero-oligomerization partner of the other SMADs. The conserved carboxy-terminal domains of SMADs are sufficient for inducing most of the ligand-specific effects, and are the primary targets of tumorigenic inactivation. We now describe the crystal structure of the C-terminal domain (CTD) of the Smad4/DPC4 tumour suppressor, determined at 2.5 Å resolution. The structure reveals that the Smad4/DPC4 CTD forms a crystallographic trimer through a conserved protein–protein interface, to which the majority of the tumour-derived missense mutations map. These mutations disrupt homo-oligomerization *in vitro* and *in vivo*, indicating that the trimeric assembly of the Smad4/DPC4 CTD is critical for signalling and is disrupted by tumorigenic mutations.

The conserved C-terminal domain can mediate many of the biological effects of SMAD proteins, and the conserved N-terminal domain can negatively regulate the SMAD activity^{14,15}. When over-expressed in a Smad4/DPC4^{–/–} cell line, the Smad4/DPC4 CTD can activate the transcription of TGF- β responsive genes and result in