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Structural Mechanism of Smad4 Recognition by the Nuclear Oncoprotein Ski: Insights on Ski-Mediated Repression of TGF-β Signaling

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Summary

The Ski family of nuclear oncoproteins represses TGF-β signaling through interactions with the Smad proteins. The crystal structure of the Smad4 binding domain of human c-Ski in complex with the MH2 domain of Smad4 reveals specific recognition of the Smad4 L3 loop region by a highly conserved interaction loop (I loop) from Ski. The Ski binding surface on Smad4 significantly overlaps with that required for binding of the R-Smads. Indeed, Ski disrupts the formation of a functional complex between the Co- and R-Smads, explaining how it could lead to repression of TGF-β, activin, and BMP responses. Intriguingly, the structure of the Ski fragment, stabilized by a bound zinc atom, resembles the SAND domain, in which the corresponding I loop is responsible for DNA binding.

Introduction

Members of the TGF-β family of extracellular growth factors regulate cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate in metazoans (Massagué, 1998; Roberts and Sporn, 1990). TGF-β signaling from the cell membrane to the nucleus is mediated by the Smad family of proteins (Heldin et al., 1997; Miyazono et al., 2000; Wrana and Attisano, 2000). Upon ligand activation, a specific receptor-regulated Smad (R-Smad) is phosphorylated by a corresponding receptor Ser/Thr kinase. For example, Smad1, Smad5, and Smad8 are phosphorylated by the BMP receptors, whereas Smad2 and Smad3 are phosphorylated by the activin/TGF-β receptors. In each case, the phosphorylated R-Smad is translocated into the nucleus and forms a functional signaling complex with the comediator Smad (Co-Smad), Smad4, to modulate expression of the ligand-responsive genes.

Because of its critical role in cell fate determination, TGF-β signaling is subject to many levels of positive and negative regulation, targeting both the receptors and the intracellular mediators (Massagué and Chen, 2000). Among the negative regulators of Smad function, c-Ski and c-SnoN are two highly conserved members of the Ski family of proto-oncoproteins. Overexpression of Ski or SnoN causes oncogenic transformation of chicken and quail embryo fibroblasts (Boyer et al., 1993; Colmenares and Stavnezer, 1989; Colmenares et al., 1991); high levels of Ski or SnoN are associated with many types of human cancer cells (Fumagalli et al., 1993; Nomura et al., 1989). Ski or SnoN antagonizes TGF-β signaling through direct interactions with the Co-Smad, Smad4, and the R-Smads, Smad2 or Smad3 (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999a, 1999b; Xu et al., 2000). In addition, Ski also represses BMP signaling through interactions with Smad4 and BMP-specific R-Smads, Smad1 or Smad5 (Wang et al., 2000). The mechanism of Ski-mediated repression of TGF-β signaling has been primarily attributed to transcriptional modulation, through recruitment of the nuclear corepressor (N-CoR) and histone deacetylase complex (HDAC) as well as interference of Smad-mediated binding to the transcriptional coactivator, p300/CBP (reviewed in Liu et al., 2001).

In response to ligand-dependent signal transduction, Ski/SnoN forms a complex with Smad4 as well as the R-Smads (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999a, 1999b; Xu et al., 2000). Ski/SnoN contains discrete and separable sequence elements necessary for binding to Smad4 or R-Smad; thus, it is able to contact both Smad4 and R-Smad simultaneously. The Smad4-Ski interaction plays a central role in the biological activities of Ski as evidenced by the observation that v-Ski, which induces potent oncogenic transformation of chicken embryo cells (Stavnezer et al., 1986; Colmenares and Stavnezer, 1990), only interacts with Smad4 but not Smad2 or Smad3 (Luo et al., 1999; J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). Furthermore, Ski failed to repress a TGFβ-responsive reporter in cells lacking Smad4 (Xu et al., 2000), again highlighting the significance of the Smad4-Ski interactions.

To reveal the mechanism of Smad4 recognition by Ski/SnoN and to understand Ski-mediated repression of TGF-β, activin, or BMP signaling, we determined the crystal structure of a complex between a conserved Smad4 binding fragment of Ski and the MH2 domain of Smad4 at 2.85 Å resolution. The structure of the Ski fragment, stabilized by a tightly bound zinc atom, resembles that of the DNA binding SAND domain. Interestingly, the L3 loop region of Smad4 is specifically recognized by a highly conserved interaction loop (I loop) from Ski, whereas the corresponding I loop in the SAND domain is critically involved in DNA binding. The Ski binding surface on Smad4 significantly overlaps with that required for binding of the phosphorylated C terminus of the R-Smad. Consequently, Ski disrupts functional complexes between the Co- and R-Smads both in vitro and in vivo.

Results

Overall Structural Features

Smad4 and Ski were found to form a 1:1 complex in solution as judged by gel filtration (data not shown).

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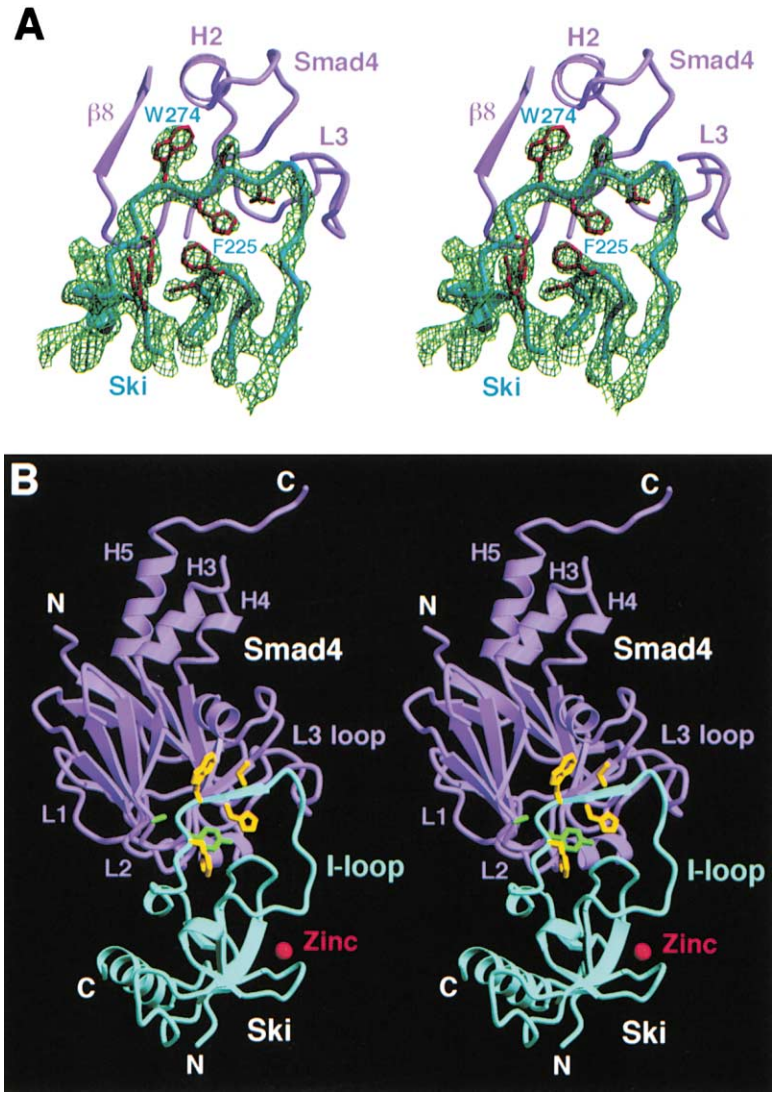

Figure 1. Overall Structure of the Smad4/Ski Complex

(A) A stereo view of the representative electron density map. The experimental $F_o - F_c$ map, shown at 1.5σ, was calculated using phases generated from the molecular replacement solution of Smad4 before modeling the Smad4-Ski interactions. The final refined model of the Ski fragment is shown in cyan, with a few prominent residues colored red.

(B) Schematic representation of the Smad4-Ski complex in stereo. Some secondary structural elements are labeled. The zinc atom, shown in red, is coordinated by Cys247, Cys250, His262, and His264. The hydrophobic residues on the interaction loop (I loop) of Ski are highlighted in yellow. All figures except Figures 3A and 5A were prepared using MOLSCRIPT (Klaulis, 1991). Figures 3A and 5A were prepared using GRASP (Nicholls et al., 1991).

Using limited proteolysis, we identified a minimal structural domain of Ski (residues 219–312) necessary and sufficient for binding to the MH2 domain of Smad4 (residues 319–552). These two interacting domains were coexpressed in bacteria, copurified as a stoichiometric complex, and cocrystallized. The structure was determined by molecular replacement and refined at 2.85 Å resolution (Figure 1 and Table 1).

The structure of Smad4 bound to Ski contains a central β sandwich, capped on one end by a three-helix bundle (helices H3, H4, and H5) and on the other end by a loop-helix region (loops L1, L2, and L3) (Figure 1B). This arrangement is highly similar to that of the Smad4 in isolation (Shi et al., 1997), with 0.73 Å root-mean-square deviation (rmsd) for 184 aligned Cα atoms (Figure 2A). Interestingly, the regions that display detectable conformational changes map exclusively to both ends of the β sandwich scaffold, as indicated by the white arrows (Figure 2A). As these regions are important for binding to the R-Smads (Shi, 2001; Wu et al., 2001), these conformational changes in Smad4 could inhibit the formation of a functional heteromeric Smad complex. In particular, loops L1 and L2, on which three residues (Asp351, Arg361, and Val370) are frequently targeted by tumor-derived missense mutations (Massagué et al., 2000; Shi et al., 1997), are in the close vicinity of Ski. Compared to Smad4 in isolation, some backbone atoms on loops L1/L2 shift over a distance of 2–3 Å upon binding to Ski, a fraction of which could ensure the disruption of the interface between Smad4 and an R-Smad.

The 1:1 Smad4-Ski complex forms a relatively elongated structure, with the Ski fragment binding to the loop-helix end of the Smad4 scaffold (Figure 2A). Except for a surface loop, the bulk of the Ski fragment adopts a compact structure, comprising four α helices and five β strands (Figures 1 and 2). Four residues in Ski, Cys247, Cys250, His262, and His264 coordinate a bound zinc atom, contributing to the structural stability. These four residues are invariant among all members of the Ski family of oncoproteins, indicating the conserved feature of zinc binding. Mutation of any of these four residues or

Table 1. Data Collection and Statistics from the Crystallographic Analysis

Data Set	Native (NSLS-X25)
Resolution (Å)	99.0–2.85
Total observations	136,326
Unique observations	22,639
Data redundancy (fold)	6.1
Data coverage (outer shell)	94.5% (61.2%)
R_{sym} (outer shell)	0.073 (0.457)
Refinement	
Resolution range (Å)	20–2.85
Number of reflections ($I > 0.5\sigma$)	20,599
$R_{working}/R_{free}$	23.1% / 28.0%
Number of atoms	4673
Number of waters	15
Rmsd bond length (Å)	0.008
Rmsd bond angles (degree)	1.578

$R_{sym} = \Sigma_h\Sigma_i|I_{h,i} - I_h|/\Sigma_h\Sigma_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry-related reflections of h.
$R = \Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$, where $F_{obs} = F_P$, and F_{calc} is the calculated protein structure factor from the atomic model (R_{free} was calculated with 5% of the reflections).
Rmsd in bond lengths and angles are the deviations from ideal values.

removal of the zinc atom using EDTA leads to structural unfolding of this fragment and consequent loss of binding to Smad4 (data not shown). Supporting the importance of zinc binding, a 17-residue deletion spanning the Cys/His-containing region in v-Ski completely abolishes its transforming activity (Zheng et al., 1997).

Structural Homology between Ski and the SAND Domain

Since this Ski fragment appears to represent a novel class of Cys_2His_2-type zinc binding module, we performed a structure-based search for its homologs in the Protein Data Bank using DALI (Holm and Sander, 1993). Interestingly, this analysis revealed one significant structural homolog (Z score ~4.8), the SAND domain of nuclear protein Sp100b (Bottomley et al., 2001), which does not bind zinc. The SAND domain (named after *S*p100, *A*IRE-1, *N*ucP41/75, *D*EAF-1) is an evolutionarily conserved sequence motif found in a number of nuclear proteins that are involved in chromatin-dependent transcriptional regulation. The SAND domain in these proteins mediates DNA binding using a conserved KDWK motif. The primary sequence of the Sp100b SAND domain is 28% identical to the Ski fragment, while their structures can be superimposed with 1.7 Å rmsd for 49 Cα atoms (Figure 2B).

Ski recognizes Smad4 at the loop-helix end of the β sandwich, primarily using an extended interaction loop (I loop) (Figure 2). This I loop contains an additional strand (β4′), which forms a parallel β sheet with the β7 strand of Smad4 through five intermolecular hydrogen bonds. Intriguingly, the same I loop in the SAND domain, which harbors the KDWK motif, is responsible for DNA binding (Figure 2B; Bottomley et al., 2001). Although some of the critical DNA binding residues in the SAND domains are conserved in Ski/SnoN, we have not been able to detect specific DNA binding by the Ski fragment (data not shown). Thus, in addition to having the same fold, Ski and the SAND domain share the same interac-

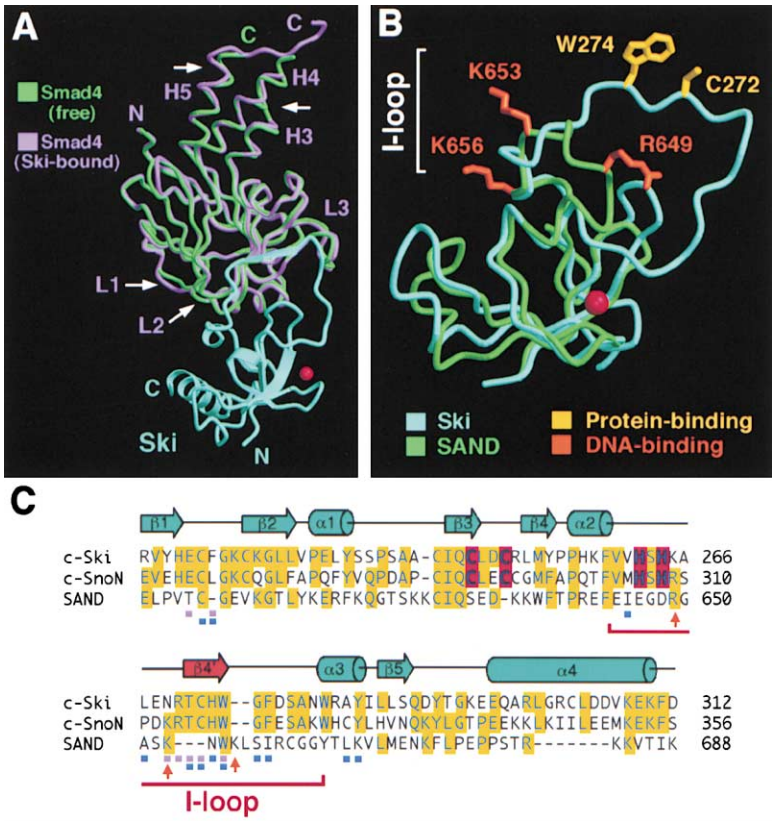

Figure 2. Structural Features of the Smad4/Ski Complex

(A) Comparison of the Ski bound Smad4 structure (purple) with the free form (green). Most conformational changes are minor and are localized to the 3-helix bundle and loop-helix regions as indicated by the arrows.

(B) Structural comparison of Ski (cyan) with the SAND domain (green). The I loop of Ski is responsible for binding Smad4, while that of the SAND domain is involved in DNA recognition. The critical residues in these activities are shown in yellow (Ski) or gold (SAND).

(C) Sequence alignment of human c-Ski and c-SnoN and the SAND domain from Sp100b. The secondary structural elements are indicated above the alignment. The β4′ strand is shown in gold to highlight the fact that it forms part of the Smad4 β sheet. The Cys/His residues that coordinate the zinc atom are shown in red. Residues that contact Smad4 through hydrogen bonds and van der Waals interactions are indicated by pink and blue squares, respectively. The three orange arrows denote key DNA binding residues in the SAND domain.

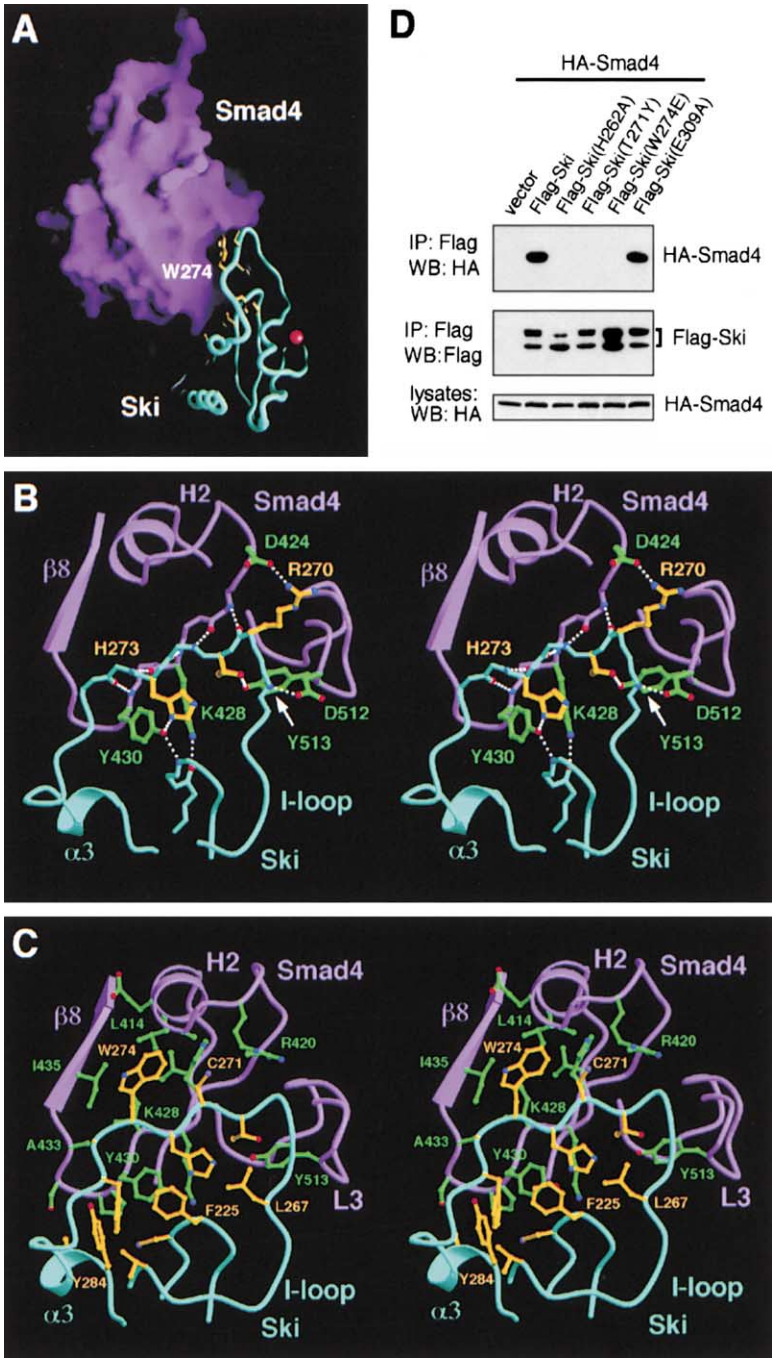

Figure 3. Recognition of Smad4 by Ski

(A) Overall view of the interactions. Smad4 (purple) is shown in its surface representation. Ski is shown as a cyan coil, while some of its interface residues are represented in yellow.

(B) A stereo view of the hydrogen bond networks at the interface. Part of the I loop forms a parallel β strand with the β7 strand of Smad4 through five intermolecular hydrogen bonds. Tyr513 of Smad4, required for binding to the phosphorylated C terminus of R-Smad, is flipped out of its normal orientation and hydrogen bonds to Thr273 of Ski. Smad4 and Ski are shown in purple and cyan, respectively. Their side chains are colored green and yellow, respectively. Hydrogen bonds are indicated by white dashed lines. Coloring scheme is the same for all other figures.

(C) A stereo view of the van der Waals contacts between Smad4 and Ski.

(D) Mutation of interface residues in Ski results in the abrogation of binding to Smad4. Flag-tagged wild-type or mutant Ski was cotransfected into 293T cells together with HA-Smad4. Cell lysate was subjected to immunoprecipitation using an anti-Flag antibody, and the Ski bound Smad4 was detected by immunoblotting using an anti-HA antibody.

tion motif, the I loop, which interacts with Smad4 and DNA, respectively. This I loop in Ski/SnoN, completely solvent-accessible and poised for binding to other cofactors, likely represents a general protein recognition motif. This hypothesis is supported by the report that the same Ski fragment also interacts with the retinoblastoma (pRB) tumor suppressor protein (Tokitou et al., 1999).

Specific Recognition of Smad4 by Ski

The specific recognition of Smad4 by Ski involves a large constellation of hydrogen bonds and van der Waals contacts, resulting in the burial of approximately 1850 Å^2 exposed surface area (Figures 2C and 3A). The loop-helix end of the Smad4 β sandwich, formed by the L3 loop, the H2 helix, and the β7/β8 strands, is intimately bound by the I loop of Ski with additional interactions from Ski residues on the short loop between strands β1 and β2 and the helix α3 (Figures 2C and 3).

In addition to the five backbone hydrogen bonds between strand β4′ on the I loop of Ski and strand β7 of Smad4, six other intermolecular hydrogen bonds specify the mutual recognition. Two of these specific contacts originate from residues on the L3 loop of Smad4, Asp512 (to Asn269 of Ski), and Tyr513 (to Thr271 of Ski) (Figure 3B). Intriguingly, Tyr513, invariant among all Smads and their homologs in *C. elegans* and *Drosophila* (Shi et al.,

1997), is also involved in coordinating the phosphorylated C terminus of R-Smad (Wu et al., 2001). In contrast to Tyr513, another important residue, Tyr430, present only in Smad4 and absent in all other Smads, makes a bifurcated contact to His273 on the I loop and the backbone amide group of Phe225 in Ski (Figure 3B).

Although hydrogen bonds appear to provide specificity in the recognition of Smad4 by Ski, massive networks of van der Waals interactions likely provide the driving force for binding (Figure 3C). In particular, the aromatic residue Trp274 on the I loop of Ski inserts into a hydrophobic pocket formed by nonpolar residues on Smad4 (Val409, Leu414, Ala418, Ile429, and Ile435). Two neighboring residues, Cys271 and Thr272, also make extensive contacts to surrounding Smad4 residues.

The I loop is highly conserved among members of the Ski family (Figure 2C). The important interface residues, Thr271, Cys272, and Trp274, are invariant among c-Ski, v-Ski, SnoN, and two other Sno-related proteins, SnoI and SnoA. Other residues involved in binding to Smad4, Cys224, Phe225, Val261, Leu267, Gly275, Phe276, and Tyr284, are either the same or substituted by conserved replacements among members of the Ski family. This analysis predicts a highly conserved binding interface between Smad4 and all members of the Ski family of proto-oncoproteins.

Mutational Analysis

To confirm the importance of the observed interactions, we generated a number of missense mutations in Smad4 or Ski and examined the interactions with their wild-type (wt) counterparts using an in vitro GST-mediated pull-down assay. In complete agreement with our structural observation, each of the four mutations in the I loop of Ski, Trp274 to Ala or to Glu, Cys272 to Tyr, and Thr271 to Tyr, led to complete abrogation of interactions with Smad4. In contrast, two control mutations in Ski, Ala266 to Tyr and Glu309 to Ala, had no impact on the interactions between Ski and Smad4 (data not shown).

Similarly, three mutations in Smad4, Tyr430 to Ala or to Asp and Ala433 to Glu, abolished binding to the Ski fragment while Ile435 to Tyr in Smad4 weakened the interaction (data not shown). Although the I loop of Ski is central to the recognition of Smad4, the isolated peptide fragment encompassing the entire I loop (residues 262–284) does not form a stable complex with the Smad4 MH2 domain, suggesting important involvement of other Smad4 binding residues in Ski. Supporting this analysis, double mutation of His222 and Glu223 to Ala in Ski renders the resulting protein completely unable to interact with Smad4 (Wang et al., 2000; J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). In the structure, both residues buttress the extensive interface between Smad4 and Ski, with Glu223 contributing an intermolecular hydrogen bond and His222 stabilizing the Ski structure. Thus, I loop only functions in the context of a properly folded structural domain.

These in vitro binding results were also confirmed in vivo in 293T cells using an immunoprecipitation assay followed by Western blot analysis (Figure 3D). The three missense mutations affecting the I loop or zinc binding failed to interact with Smad4 (lanes 3–5), while the Glu309 to Ala mutant exhibited the same level of strong interactions with Smad4 as the wt Ski (lanes 2 and 6).

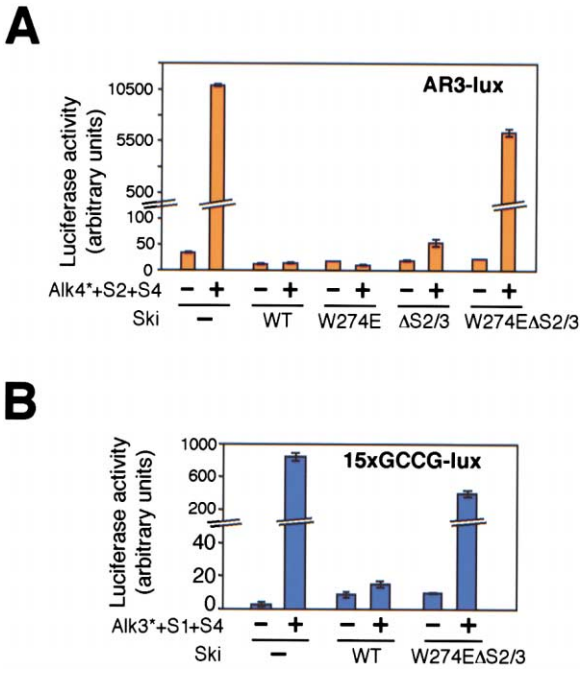

Figure 4. Importance of the Smad4-Ski Interaction

The interaction between Ski and Smad4 is essential to Ski-mediated repression of activin (A) or BMP (B) signaling. Hep3B cells were transfected with AR3-lux or 15xGCCG-lux together with the Ski and Smad proteins and constitutively active activin (Alk4*) or BMP (Alk3*) type I receptors, as described in Experimental Procedures. Luciferase activity was measured 48 hr after transfection. The ΔS2/3 Ski mutant, containing residues 24–728 of c-Ski, is deficient in binding to the R-Smads. The W274E mutant cannot interact with Smad4. A mutant Ski protein that retains binding to either Smad4 (ΔS2/3) or Smad2 (W274E) is able to repress activin signaling. In contrast, double mutation in Ski results in the abrogation of Ski-mediated repression of activin and BMP responses.

To assess the effects of disrupting the Smad4-Ski interaction on Smad-mediated transcriptional activation, we examined the ability of wt and mutant Ski proteins to repress transcription from an activin (AR3-lux, Figure 4A) or BMP (15xGCCG-lux, Figure 4B) responsive promoter. As shown before (Wang et al., 2000), binding of Ski to either the R-Smads (by Ski-W274E) or Smad4 (by Ski-ΔS2/3) is sufficient for the repressive activity of Ski, as mutations abolishing one of the Smad binding sites did not significantly affect the Ski-mediated repression of the reporter assays (Figure 4A). In contrast, this repression was completely abolished by double mutations in the I loop and in the R-Smad binding region (W274EΔS2/3) (Figure 4). These results demonstrate that both the Ski-Smad4 and Ski-Smad2/3 interactions are important for the repression of Smad-mediated transcriptional activation.

Disruption of a Smad Heteromeric Complex by Ski In Vitro

The Ski binding surface on Smad4 involves the L3 loop region that is responsible for binding to the phosphorylated C terminus of an R-Smad (Wu et al., 2001). In particular, Tyr513 of Smad4, required for binding to the phosphorylated Ser465 (pSer465) of Smad2 (Wu et al.,

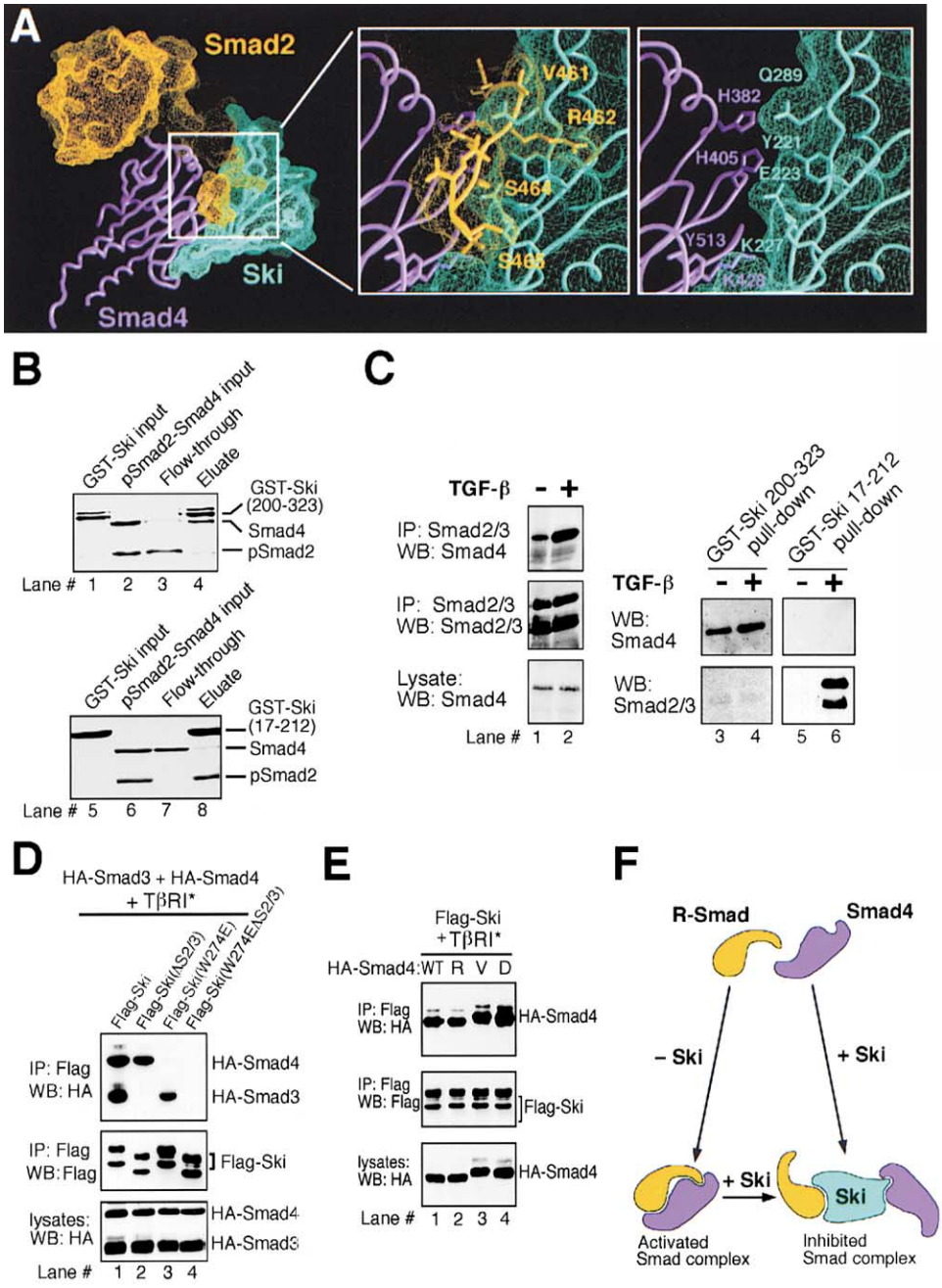

Figure 5. Ski Disrupts a Functional Complex between Smad4 and R-Smads

(A) The Smad2 binding surface on Smad4 overlaps significantly with that required for binding to Ski. Smad4 is shown in purple, while Smad2 and Ski are represented by yellow and cyan surface mesh, respectively. Binding of the phosphorylated C terminus of Smad2 to the basic loop-strand region of Smad4 causes severe steric clash between Smad2 and the Ski fragment. For comparison, the interface between Smad4 and Ski is shown in the right image.

(B) Ski disrupts a Smad2-Smad4 complex in vitro. In the top gel, GST-Ski (200–323), which only binds to Smad4, was immobilized on glutathione resin, and a stoichiometric pSmad2-Smad4 complex was passed through the resin. The eluate contains only the Smad4 moiety. In the bottom gel, the same experiment was performed using GST-Ski (17–212), which only interacts with Smad2.

(C) Ski disrupts an endogenous R-Smad-Smad4 complex. Left gel: The endogenous Smad4 forms a heteromeric complex with Smad2/Smad3 upon TGFβ treatment. Endogenous Smad2 and Smad3 were isolated from untreated (lane 1) or TGFβ-treated (lane 2) Hep3B cells by immunoprecipitation using an anti-Smad2/3 antibody, and the associated Smad4 was detected by Western blotting using anti-Smad4. Right gel: GST-Ski (200–323) only bound to Smad4 but failed to pull down Smad2 or Smad3. Immobilized GST-Ski fragment was incubated with untreated (lane 3) or TGFβ-treated (lane 4) Hep3B cell lysates. Smad proteins bound to GST-Ski were eluted and detected by Western blotting with specific antibodies as indicated. In similar experiments, GST-Ski (17–212) only interacted with Smad2/Smad3 upon TGFβ treatment, but failed to bind to Smad4 (lanes 5 and 6).

(D) Ski disrupts a Smad3-Smad4 complex in vivo. Wild-type or mutant Flag-Ski was transfected into 293T cells together with constitutively active TβRI (TβRI*), HA-Smad3, and HA-Smad4. Smad proteins associated with Ski were isolated by immunoprecipitation using an anti-Flag

2001), is hydrogen bonded to Thr271 on the l loop of Ski (Figure 3B). A detailed analysis of this binding surface reveals that both the backbone and the side chains of a number of important interface residues from Ski would sterically clash with the Smad4 bound phosphorylated C terminus of R-Smad, such as Smad2 or Smad3 (Figure 5A). In the modeling analysis, three Smad2 residues, Arg462, Ser464, and pSer465, significantly overlap with Ski and penetrate into the Ski structure (Figure 5A). In addition, binding of Ski to Smad4 also causes some conformational change in the loop-helix region (Figure 2A) that may adversely affect the formation of a heteromeric complex between Smad4 and R-Smad. These structural findings strongly suggest that Ski/SnoN and R-Smad compete for binding to Smad4 and further predict that Ski/SnoN may disrupt or prevent the formation of a functional complex between Smad4 and the R-Smad in the nucleus.

To confirm this structure-based conclusion, we investigated the interactions between Ski/SnoN and a Smad2-Smad4 complex in vitro. First, we used a Ski fragment (residues 200–323) that only binds to Smad4. If Ski does not disrupt the Smad2-Smad4 interaction, then a GST fusion protein with this Ski fragment is expected to pull down the heteromeric Smad complex rather than just one component. To perform this experiment, we prepared and characterized a stoichiometric complex between Smad4 and the phosphorylated Smad2 (pSmad2) using gel filtration (Wu et al., 2001). However, when this complex was applied to the immobilized Ski fragment (Figure 5B, lane 2), the flow-through fraction contained mainly pSmad2 (lane 3), and only Smad4 was retained in a complex with Ski (lane 4), indicating disruption of the pSmad2-Smad4 heteromeric complex by Ski. Consistent with our structural analysis (Figure 2A), Ski is also able to disrupt the unphosphorylated Smad2-Smad4 complex in vitro (data now shown).

Although how Ski/SnoN binds to R-Smad such as Smad2 remains structurally uncharacterized, it is possible that Ski/SnoN-mediated binding to R-Smad also interferes with the formation of a heteromeric Smad complex, as this activity can maximize the Ski-mediated repression of TGF-β signaling. To examine this possibility, we used a Ski fragment (residues 17–212) that only interacts with R-Smad to perform the binding assay (Figure 5B, bottom gel). The result revealed that Ski-mediated binding to pSmad2 also leads to significant disruption of the heteromeric complex between pSmad2 and Smad4 (lanes 7 and 8). This Ski fragment is also capable of disrupting the unphosphorylated Smad2-Smad4 complex in vitro (data not shown). In addition, the corresponding SnoN fragment (residues 81–256) also exhibits the same activity (data not shown).

Disruption of a Smad Heteromeric Complex by Ski In Vivo

To show that Ski or SnoN can also disrupt the endogenous Smad complex formed in response to TGFβ, the same GST-Ski fragments were incubated with cell lysates derived from TGFβ-stimulated or untreated Hep3B cells. Smad proteins bound to the GST-Ski fragments were then eluted and analyzed by Western blotting using anti-Smad2/3 or anti-Smad4 antibodies. As shown before, TGFβ induces the formation of a heteromeric R-Smad/Smad4 complex (Figure 5C, lane 2). Consistent with our structural prediction and results from in vitro assays, GST-Ski (200–323) is only capable of pulling out Smad4, but not Smad2 or Smad3 in both untreated and TGFβ-treated cell lysates (lanes 3 and 4). In a parallel experiment, GST-Ski (17–212) interacted only with Smad2 or Smad3 in a TGFβ-dependent manner, but not with Smad4 (lanes 5 and 6). The same conclusion was reached using the corresponding SnoN fragments (data not shown). These results indicate that Ski/SnoN can disrupt an endogenously formed heteromeric Smad complex through binding to either the R-Smad or Smad4.

The ability of Ski to disrupt an R-Smad/Smad4 complex in vivo is also demonstrated by a coimmunoprecipitation assay in 293T cells cotransfected with a constitutively active TβRI (T204D), HA-Smad3, HA-Smad4, and Flag-tagged Ski. While wt Ski interacted with both Smad3 and Smad4 (Figure 5D, lane 1), a Ski fragment (ΔS2/3, residues 24–728), which is deficient in binding to Smad3, only associated with Smad4 (Figure 5D, lane 2). Similarly, the mutant Ski (W274E), unable to interact with Smad4, only bound to Smad3 (lane 3). These results again demonstrate that Ski does not interact with a heteromeric Smad3/Smad4 complex. Rather, Ski disrupts such a complex by simultaneously interacting with both of its components.

If Ski disrupts the functional Smad heteromeric complexes, then the Ski-Smad4 interaction should not require the formation of the heteromeric complex between the R-Smad and Smad4. Indeed, three Smad4 mutants defective in the formation of heteromeric Smad complexes (Wu et al., 2001) all interacted with Ski as efficiently as the wt Smad4 (Figure 5E). Thus, Ski can bind to the R-Smad and Smad4 independently through two separate regions (Wang et al., 2000).

Taken together, our biochemical analyses confirm the structural conclusion that Ski disrupts a heteromeric complex between Smad4 and R-Smad through binding to either component. Although Smad4 and Smad2/Smad3 still remain bound simultaneously to the wild-type Ski/SnoN protein through two separate sets of interactions (Figure 5F), they are no longer in an active conformation that stimulates gene expression. Thus,

antibody and detected by Western blotting using an anti-HA antibody. A Ski fragment deficient in binding to Smad3 (ΔS2/3, residues 24–728) only interacted with Smad4, while another Ski fragment deficient in binding to Smad4 (W274E) only interacted with Smad3.

(E) Heterooligomerization of Smad4 with the R-Smads is not required for the interaction of Smad4 with Ski. HA-tagged wt or mutant Smad4 was transfected into 293T cells together with constitutively active TβRI (TβRI*) and Flag-Ski. Smad4 associated with Ski was isolated by immunoprecipitation using an anti-Flag antibody and detected by Western blot using an anti-HA antibody. Mutants: R, R361C; V, V370D; D, D537E.

(F) Schematic diagram of a proposed mechanism on the Ski-mediated repression of TGF-β signaling. Ski-mediated simultaneous binding to the Co- and R-Smads prevents the formation of a heteromeric Smad complex required for TGF-β, activin, and BMP signaling.

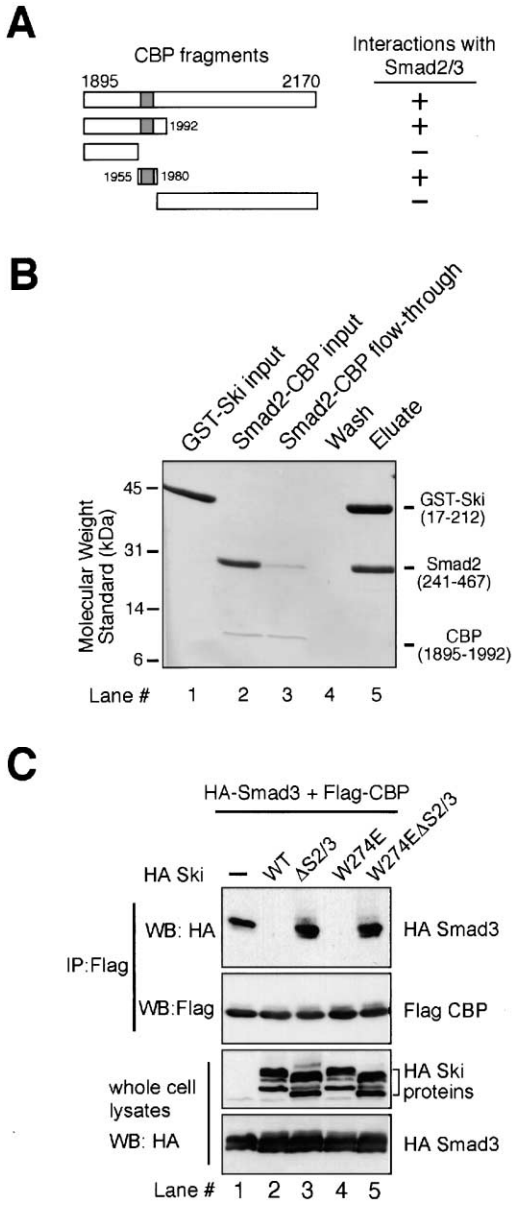

Figure 6. Dissociation of the Transcription Coactivator CBP/p300 from R-Smads through Ski/SnoN Binding

(A) Mapping of a minimal sequence motif in CBP that interacts with Smad2/Smad3. These CBP fragments were generated as fusion proteins with GST. A glutathione resin-mediated pull-down assay was used to assess the interaction between these CBP fragments and the MH2 domains of Smad2. The gray box indicates a predicted amphipathic helix in CBP.

(B) Disruption of a Smad2-CBP complex by Ski in vitro. GST-Ski (17–212), which only binds to Smad2, was immobilized on glutathione resin, and a stoichiometric Smad2-CBP complex was passed through the resin. Disruption of the Smad2-CBP complex is evident in the flow-through fraction (lane 3). The eluate contains only the Smad2 moiety (lane 5).

(C) Disruption of a Smad3-CBP complex by Ski in vivo. Flag-CBP (1892–2170) was transfected into 293T cells together with HA-Smad3, a constitutively active type I TGFβ receptor and various Ski mutants as indicated. Smad3 associated with the CBP fragment was isolated by immunoprecipitation using an anti-Flag antibody and detected by Western blotting using an anti-HA antibody. The wt Smad3 forms a stable complex with CBP (1892–2170) (lane 1). Wild-type or W274E Ski can disrupt this complex (lanes 2 and 4), while the mutant Ski, deficient in binding to the R-Smad (ΔS2/3) or to all Smads (W274EΔS2/3), failed to do so (lanes 3 and 5).

binding of Ski/SnoN to the Smads converts the Smad heteromeric complex into an inactive conformation. In this way the Ski family of proto-oncoproteins can negatively regulate Smad activity in the nucleus. Similarly, Ski negatively regulates BMP signaling (Wang et al., 2000) through disruption of a functional Smad1-Smad4 complex.

Disruption of a Smad-CBP Complex by Ski

The Smad proteins directly interact with the nuclear coactivator CBP/p300 to activate the transcription of TGFβ-responsive genes (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Topper et al., 1998). Thus, one of the functional consequences of inactivation of the Smad complex by Ski may be the disruption of the Smad/CBP interaction. Indeed, Ski was shown to interfere with the interaction of Smad3 with CBP/p300 in vivo (Akiyoshi et al., 1999).

To further confirm these results, we carried out in vitro binding assays. Previous studies show that the MH2 domain of the R-Smads and a C-terminal fragment (1891–2175) of CBP are responsible for the Smad-CBP interactions (Janknecht et al., 1998). Using an in vitro GST-mediated pull-down assay, we further identified a 25-residue sequence motif of CBP/p300 (1955–1980) that is both necessary and sufficient for the formation of a stable complex with the R-Smads (Figure 6A). Interestingly, this sequence motif is predicted to adopt an amphipathic α helix by the program DNAstar. Using gel filtration, we generated a stoichiometric complex between the MH2 domain of Smad2 and a CBP fragment (1895–1992). Then, we examined the effect of Ski/SnoN-mediated binding to Smad2 on this complex (Figure 6B). As anticipated, the immobilized GST-Ski (17–212) significantly disrupted the Smad2-CBP complex as judged by the decreased intensity of the Smad2 protein in the flow-through fraction (lane 3). Consequently, the eluate contains a stable complex between GST-Ski and Smad2 (lane 5). The same result was obtained for the corresponding SnoN fragment (81–256) (data not shown).

To further investigate whether the ability of Ski to interfere with the CBP-R-Smad interaction correlates with its ability to interact with the Smad proteins, wild-type as well as various Ski mutants were cotransfected into 293T cells together with Flag-CBP (1892–2170) and HA-Smad3. In the absence of Ski, the CBP fragment readily interacted with Smad3 upon activation of TGFβ signaling (Figure 6C, lane 1). As reported previously (Akiyoshi et al., 1999), wt Ski disrupted this interaction (lane 2). In contrast, expression of the mutant Ski deficient in binding to R-Smad (ΔS2/3) or to both R-Smad and Smad4 (W274EΔS2/3) had no effect on the CBP-Smad3 interaction (lanes 3 and 5), indicating that the interaction of Ski with the R-Smad is responsible for the dissociation of CBP from the Smad proteins. Supporting this conclusion, the W274E mutant, which can disrupt the Smad3-Smad4 heteromeric complex but retains binding to Smad3, still blocked binding of CBP to Smad3 (lane 4).

This result demonstrates that the dissociation of CBP/p300 from Smad3 is due to displacement by Ski, but not due directly to the disruption of the Smad complex.

Discussion

The Ski family of proto-oncoproteins uses two separate sequence motifs to interact with the Co-Smad, Smad4, and the R-Smads, Smad2, Smad3, Smad1, or Smad5 (Wang et al., 2000; J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). Binding of Ski to Smad4 plays an essential role in Ski-mediated repression of TGF-β signaling as well as Ski-mediated transforming activity (J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). For example, a conserved Ski fragment (residues 76–304), which binds to Smad4 but does not interact with Smad2 or Smad3, is necessary and sufficient for the transforming activity of Ski (Zheng et al., 1997). v-Ski, the transforming protein of the avian Sloan Kettering virus, interacts only with Smad4, but not R-Smads (Luo et al., 1999; J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). Mutation of the Smad4 binding site in v-Ski significantly impairs its transforming activity (J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data). These results support a critical role of Ski-Smad4 interaction in the repression of TGFβ signaling by Ski.

Analysis of the crystal structure of a Ski fragment bound to Smad4 indicates that Ski and the R-Smads compete for mutually exclusive binding to Smad4, leading to the disruption of functional Smad4-R-Smad complexes. Consistent with this analysis, we found that the interaction of Ski with Smad4 does not require the formation of a heteromeric complex with the R-Smads. Consequently, the high level of Ski protein present in many tumor cells could bind to Smad4 constitutively and prevent it from forming an active complex with the R-Smad. This disruption or inhibition of the Smad heteromeric complex by Ski could affect the interaction of the Smad proteins with transcriptional coactivators or the proper assembly of a Smad-containing transcription initiation complex. Additionally, since Smad4 contributes to the cooperative DNA binding as exhibited by the heteromeric Smad complex (Denissova et al., 2000; Shi et al., 1998; Shi, 2001) and by the fact that Smad3 does not bind to the Smad Binding Element (SBE) in the absence of Smad4 in vivo (Denissova et al., 2000), dissociation of Smad4 from the R-Smads eliminates this cooperation and may weaken the Smad-promoter interaction. Thus, Ski/SnoN represses TGFβ signaling by multiple mechanisms. In addition to recruitment of a transcriptional repressor complex and dissociation of the transcriptional coactivator p300/CBP from the Smads (Akiyoshi et al., 1999; Luo et al., 1999), Ski/SnoN also disrupts a functional Smad heteromeric complex, inactivating its ability to activate transcription. To the best of our knowledge this seems to represent a novel mechanism for the negative regulation of Smad activity in the nucleus by the Ski family of proto-oncoproteins. Furthermore, since Ski/SnoN-mediated binding to Smad4 or the R-Smads prevents the formation of a functional heteromeric Smad complex, Ski/SnoN effectively sequesters the Smad proteins in an inactive conformation. This scenario may contribute to Ski-mediated downregulation of Smad activity both in the absence and in the presence of TGF-β signaling.

In this study, important conclusions were derived from the use of Ski mutants, such as the double mutation W274EΔS2/3. These mutations are unlikely to disrupt the overall folding of the Ski protein, because the W274E mutation occurs in the completely solvent-exposed I loop and the ΔS2/3 mutation involves the removal of the N-terminal 23 amino acids that are predicted to be modular to the rest of the sequences. In addition, these Ski mutants are as stable as the wt protein and interact normally with other proteins (J. He, S. Tegen, A. Krawitz, G.S. Martin, and K.L., unpublished data).

Our structural characterization demonstrates that this essential Ski fragment forms a novel Cys_2His_2-type zinc binding module. The bound zinc atom plays an indispensable role in stabilizing the three-dimensional fold of this Ski domain, as mutation of a single zinc-coordinating residue (His262 to Ala) results in complete abrogation of interactions with Smad4 (Figure 3D). The significance of zinc binding in this essential domain is further manifested by the complete loss of transforming activity for a v-Ski mutant containing a deletion of the zinc binding residues (Zheng et al., 1997).

A structure-based search of the Protein Data Bank (PDB) identified the SAND domain as a structural homolog of the Smad4 binding domain of Ski. The SAND domain represents a large family of DNA binding motifs present in transcription factors and other nuclear proteins. Both Ski and the SAND domain use a common structural motif, the I loop, to interact with their partners, protein (Smad4) or DNA. This comparison raises an interesting possibility that this Ski fragment may have direct DNA binding activity. However, we failed to detect any direct binding of this Ski fragment to the SBE (data not shown). Although we cannot rule out the possibility that Ski may directly contact other DNA sequences, the lack of the critical DNA binding KDWK motif suggests that the I loop in Ski is primarily a protein binding segment and not a DNA binding motif. Thus, similar to the RNA recognition motif (RRM) sometimes serving as a protein-protein interaction domain (Kielkopf et al., 2001; Sakashita and Sakamoto, 1996), the SAND domain is another example of a conserved motif that mediates both nucleic acid binding and protein-protein interaction.

Our structural analysis also explains why this conserved region in Ski (200–323) only recognizes the Co-Smad but not the R-Smad. Although some Ski binding residues (Tyr513 and Asp512) from the L3 loop of Smad4 are conserved in Smad2 and Smad3, two of the key binding residues, Tyr430 and Ala433, are unique to Smad4 and are replaced by dissimilar residues (Pro and Gly) in the R-Smads (Shi et al., 1997). In fact, this part of the binding surface is among the most divergent between Smad4 and R-Smads (Shi et al., 1997). Hence, this Ski fragment is only specific for Smad4 and does not interact with any other Smads.

While this paper was in revision, another manuscript was published, which suggested that a 28-residue Ski fragment is able to interact with a functional heteromeric Smad complex (Qin et al., 2002). This proposed interaction is biologically irrelevant, as the full-length Ski protein disrupts any such Smad complexes and thus is

unable to bind the heteromeric Smad complex in its functional form (Figure 5).

In summary, our study provides a structural mechanism for Ski/SnoN binding to the Co-Smad and reveals how disruption of a heteromeric Smad complex by the proto-oncoprotein Ski/SnoN can lead to repression of TGF-β signaling. As the Ski family of proteins contributes to cellular transformation and tumorigenesis, our structural study may also aid in the rational design of Ski-antagonists.

Experimental Procedures

Protein Preparation

All constructs were generated using a standard PCR-based cloning strategy, and the identities of individual clones were verified through double-stranded plasmid sequencing. For crystallization, the Smad4 binding fragment of Ski (219–312) and Smad4 MH2 domain (319–552) were cloned into the vectors pGEX-2T (Amp^R, Pharmacia) and pBB75 (Kan^R), respectively, and coexpressed in *E. coli* strain BL21(DE3). The Smad4/Ski complex was purified using glutathione sepharose 4B resin as described (Chai et al., 2000). For in vitro biochemistry, the recombinant wild-type and mutant Smad4 and Ski fragments were overexpressed as GST-fusion proteins using pGEX-2T (Pharmacia). Phosphorylated Smad2/Smad4 complexes were prepared as described (Wu et al., 2001).

Luciferase Reporter Assay

To assay for BMP response, Hep3B cells were cotransfected with 0.4–0.75 μg of wt or mutant Ski, 0.75 μg of 15xGCCG-Lux, 0.5 μg of constitutively active BMP type I receptor (Alk3*), 0.25 μg of Smad1, and 0.25 μg of Smad4. To assay for activin response, Hep3B cells were cotransfected with 0.4–0.75 μg of wt or mutant Ski, 0.75 μg of AR3-Lux, 0.5 μg of constitutively active activin type I receptor (Alk4*), 0.25 μg of Smad2, 0.25 μg of Smad4, and 0.01 μg of FAST1. Cells were cultured in serum-free medium for 24 hr after transfection, and luciferase activity was measured after 48 hr.

Immunoprecipitation and Western Blotting

Flag-Ski was isolated from transfected 293T cells by immunoprecipitation with an anti-Flag antibody (SIGMA) followed by elution with Flag peptide as described (Zhou et al., 1998; Stroschein et al., 1999). Association of HA-Smad4 with Ski was analyzed by immunoblotting of anti-Flag precipitates with anti-HA. To control for equal loading of Ski proteins, the membrane was reblotted with anti-Flag. To detect endogenous R-Smad/Smad4 complex, Hep3B cells were either unstimulated or stimulated with 200 pM TGF-β for 1 hr. Endogenous Smad2 and Smad3 were isolated by immunoprecipitation with anti-Smad2/3 antibodies (Santa Cruz Biotechnology), and the associated Smad4 was detected by Western blotting with anti-Smad4 antibody.

For the Smad3-CBP association, HA-tagged wt or mutant Ski was cotransfected into 293T cells together with HA-tagged Smad3, Flag-tagged CBP (1892–2170), and constitutively active type I TGFβ receptor (T204DTβRI). The CBP-Smad3 complexes were isolated by immunoprecipitation using an anti-Flag antibody and eluted with the Flag peptide. Association of Smad3 with CBP was demonstrated by immunoblotting anti-Flag precipitates with an anti-HA antibody. To control for equal loading of CBP proteins, the membrane was reblotted with an anti-Flag antibody.

GST-Mediated Pull-Down Assay

For the in vitro experiments, approximately 0.4 mg of a recombinant Ski (200–323) or Ski (17–212) fragment was bound to 200 μl of glutathione resin as a GST-fusion protein. The resin was washed four times with 400 μl buffer to remove excess unbound protein. Then, 600 μg of a Smad2-Smad4 complex was allowed to flow through the resin. After extensive washing with an assay buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM dithiothreitol (DTT), the bound proteins were eluted with 5 mM reduced glutathione and visualized by SDS-PAGE with Coomassie staining. For isolation of endogenous Smad complexes, cell lysates prepared from Hep3B cells that were either unstimulated or stimulated with 200 pM TGF-β1 for 1 hr were incubated with 10 μg immobilized GST-Ski fragments. The Smad proteins associated with the Ski or SnoN fragments were eluted with reduced glutathione and detected by Western blot using antibodies that recognize Smad4 or Smad2/3.

Crystallization and Data Collection

Crystals were grown by the hanging-drop vapor-diffusion method by mixing the Smad4/Ski complex (~15 mg/ml) with an equal volume of reservoir solution containing 100 mM HEPES (pH 7.5), 10% 1,4-dioxane (v/v), and 900 mM K_2HPO_4/KH_2PO_4 (at pH 7.0). Small crystals appeared after 1–4 days and were used for macroseeding to generate larger crystals. The crystals, with a typical dimension of 0.2 × 0.2 × 0.6 mm^3, belong to the spacegroup $P3_221$, with a = b = 109.8 Å and c = 141.1 Å. There are two complexes per asymmetric unit. Crystals were equilibrated in a cryoprotectant buffer containing reservoir buffer plus 20% glycerol (v/v) and were flash frozen in a cold nitrogen stream at −170°C. The native data set was collected at beamline X25 at the National Synchrotron Light Source and processed using the software Denzo and Scalepack (Otwinowski and Minor, 1997).

Structure Determination

The structure of the Smad4/Ski complex was determined by molecular replacement, using the software AMORE (Navaza, 1994). The coordinates of Smad4 (PDB code 1ygs) were used for rotational research against all reflections between 15 and 3.0 Å in the 2.85 Å data set. The top 50 solutions from the rotational search were individually used for a subsequent translational search, which yielded two excellent solutions. This model was examined with the program O (Jones et al., 1991). Refinement by the program CNS (Brunger et al., 1998), against the 2.85 Å native data set, allowed visualization of the Ski fragment. A model was built with the program O and refined further by simulated annealing. The final atomic model contains two complexes of the Smad4/Ski complex. One complex contains Smad4 residues 319–452 and 492–550, Ski residues 216–312, and one zinc atom. The other complex contains Smad4 residues 319–461 and 492–543, Ski residues 217–312, and one zinc atom. Since these two complexes exhibit identical features, we only discuss one such complex in the text (Figure 1B).

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Accession Numbers

Atomic coordinates for the Smad4/Ski complex have been deposited with the Protein Data Bank (accession number 1MR1).