The transforming activity of Ski is dependent on its ability to repress the activity of Smad proteins

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Summary

The regulation of cell growth and differentiation by the transforming growth factor-β is mediated by the Smad proteins. In the nucleus, the Smad proteins are negatively regulated by two closely related nuclear proto-oncoproteins, Ski and SnoN. When overexpressed, Ski and SnoN induce oncogenic transformation of chicken embryo fibroblasts. However, the mechanism of transformation by Ski and SnoN has not been defined. We have previously reported that Ski and SnoN interact directly with Smad2, Smad3 and Smad4 and repress their ability to activate TGFβ target genes through multiple mechanisms. Since Smad proteins are tumor suppressors, we hypothesized that the ability of Ski and SnoN to inactivate Smad function may be responsible for their transforming activity. Here we show that the R-Smads, Smad2 and Smad3, and the co-Smad, Smad4, bind to different regions in Ski or SnoN. Mutation of both regions, but not each region alone, markedly impaired the ability of Ski and SnoN to repress TGFβ-induced transcription activation and cell cycle arrest. Moreover, when expressed in chicken embryo fibroblasts, mutant Ski or SnoN defective in binding to the Smads failed to induce oncogenic transformation. These results suggested that the ability of Ski and SnoN to repress the growth inhibitory function of the Smads is required for their transforming activity. This may account for the resistance to TGFβ-induced growth arrest in some human cancer cell lines that express high levels of Ski or SnoN.

Introduction

ski and *sno* are members of the *ski* family of nuclear proto-oncogenes (1-3). Ski was originally identified as the transforming protein of the avian Sloan-Kettering retrovirus (v-Ski) (4,5). The human cellular homologue c-*ski* and the closely related *snoN* were later cloned based on sequence homology with v-*ski* (1). The amino-terminal region of Ski is highly conserved among Ski family members including v-Ski (more than 95% sequence identity) and SnoN (70% identity), while the carboxyl terminal half of the molecule shows little homology among the family members. The N-terminal homology region is necessary and sufficient for the known biological activities of Ski and SnoN (6,7). Compared to c-Ski, v-Ski is truncated at the carboxyl terminus (5,8). However, this truncation does not appear to be responsible for the activation of *ski* as an oncogene because overexpression of wild-type c-Ski results in oncogenic transformation of chicken and quail embryo fibroblasts (9). Thus, the transforming activity of Ski is likely due to overexpression, not truncation, of the c-Ski protein. Consistent with this notion, an elevated level of c-Ski or c-SnoN was detected in many human tumor cell lines derived from neuroblastoma, melanoma, breast cancer and carcinomas of the stomach, chorion, thyroid and epidermoid (1,10,11). In addition to upregulation of *ski* expression, mis-localization of Ski may also contribute to malignant progression. Ski was found to be present in the nucleus in cells derived from normal skin or early-stage tumors, but localized in the cytoplasm in highly malignant melanoma cells (11). However, the mechanism of transformation by Ski and SnoN has not been defined.

Ski and SnoN are incorporated into the histone deacetylase (HDAC1) complex through binding to the nuclear hormone receptor co-repressor N-CoR and mSin3A and mediate transcriptional repression of the thyroid hormone receptor, Mad and pRb (12,13). Ski

has also been shown to interact with the retinoic acid receptor in a ligand-independent manner, as well as with pRb and another transcription factor Skip (14,15). However, the role of these interactions in transformation by Ski or SnoN remains to be determined.

We and others have recently shown that Ski and SnoN interact with the Smad proteins to negatively regulate transforming growth factor-β (TGFβ) or bone morphogenic protein (BMP) signaling (16-21). Smad proteins are critical components of the TGFβ signaling pathways (22,23). In the absence of TGFβ, the two highly homologous R-Smads, Smad2 and Smad3, are distributed mostly in the cytoplasm (24-26). Upon ligand binding, the activated type I TGFβ receptor kinase phosphorylates the R-Smads, allowing them to translocate into the nucleus (25-31) and form heteromeric complexes with Smad4 (32-35). In the nucleus, the Smad complexes interact with various cellular partners and participate in diverse downstream activities. The Smads can bind to the TGFβ-responsive promoter DNA either directly or in conjunction with other sequence-specific DNA binding proteins (36). Through the C-terminal Mad homology-2 (MH2) domains, the Smad proteins interact with general or promoter-specific transcriptional co-activators and co-repressors to regulate the transcription of various TGFβ target genes (36). The Smad proteins play a central role in mediating the growth inhibitory response of TGFβ by activating the expression of Cdk inhibitors such as $p21^{CIP1}$ and $p15^{INK4B}$ (37-40). Because of this, the Smad proteins are considered important tumor suppressors. Inactivation of the Smad proteins either by deletion or by mutation has been found to accompany the malignant progression of many human cancer cells (41). In addition, Smad proteins can also be inactivated through interaction with other proteins (16-20,42,43).

Interaction of Ski or SnoN with the Smad proteins results in disruption of an active heteromeric Smad complex (16,44), displacement of transcriptional co-activator p300/CBP from the Smads (44) and recruitment of the nuclear hormone receptor co-repressor N-CoR (17,18). Through these mechanisms, Ski and SnoN repress the ability of the Smad proteins to mediate TGFβ-induced cell cycle arrest. Since the Smad proteins are tumor suppressors, we hypothesized that this ability of Ski or SnoN to inactivate Smad function may be responsible for their transforming activity. To test whether the interaction of Ski/SnoN with the Smads is indeed responsible for the transcriptional repression of the Smads and for the oncogenic activity of Ski and SnoN, we carried out biochemical and structural analyses to map the Smad-binding sites in Ski and SnoN. We recently solved the crystal structure of a Ski fragment bound to Smad4 MH2 domain (44). Based on this structure and on our mutational analysis, we have identified the amino acid residues in Ski and SnoN that mediate interaction with the Smad proteins. This has allowed us to determine whether Ski/SnoN mutants defective in binding to the Smad proteins can still repress Smad function and induce oncogenic transformation of chicken embryo fibroblasts.

Experimental procedures:

Cells, antisera and constructs:

Hep3B, a human hepatoma cell line (ATCC), was maintained in MEM supplemented with 10% fetal bovine serum (FBS). 293T and Phoenix-Eco cells were maintained in DMEM supplemented with 10% FBS. Ba/F3, a pro-B cell line, was maintained in RPMI supplemented with 10% FBS and 10% WEHI cell-conditioned medium as a source of interleukin-3 (45). Primary cultures of chicken embryo fibroblast (CEF) were prepared from 10-day-old embryos and cultured as described (46,47). CEF were maintained in DME/F10 supplemented with 2% tryptose phosphate broth (TPB), 1% chicken serum and 1% bovine calf serum.

Antiserum against N-CoR (sc-1609) was purchased from Santa Cruz Biotechnology. Anti-Flag (M2) was purchased from Sigma. Alexa fluro® 488 goat anti mouse IgG (H+L) was purchased from Molecular Probes.

HA-tagged full-length Smad2, Smad3, Smad4 and Flag-tagged full-length or truncated Ski and SnoN were subcloned into pCMV5B as described previously (17,18). Flag-tagged mutants of Ski and SnoN were generated by PCR, subcloned into pCMV5B, pBABEpuro (17,18) or helper-free RCAS.BP type A avian retroviral vector (48).

Transfection and retroviral infection:

293T and Hep3B cells were transiently transfected using the Lipofectamine Plus Protocol (Gibco BRL). To generate stable Ba/F3 cell lines overexpressing Ski or SnoN, Flag-tagged wild type and mutant Ski and SnoN in the retroviral vectors, pBABEpuro or pMX-IRES-GFP, were transfected into Phoenix-Eco packaging cells to generate retroviruses.

48 hrs after transfection, 2.8 ml viral supernatant was collected and added to $5x10^5$ Ba/F3 cells in presence of 6 μg/ml polybrene. Following centrifugation at 1000 rpm for 2 hrs at 37°C, the cells were resuspended in 2 ml RPMI complete media and co-cultivated with the transfected Phoenix-Eco packaging cells. 24 hours later, the Ba/F3 cells were removed from the Phoenix-Eco cells and cultured in fresh RPMI complete media for an additional 24 hrs. The infected cells were selected in RPMI medium containing 2 μg/ml puromycin (for pBABEpuro) or by FACS based on expression of the green fluorescence protein (GFP, for pMX-IRES-GFP)(Sigma).

Immunoprecipitation and Western Blotting:

Flag- and HA-tagged proteins were isolated from transfected 293T cell lysates by immunoprecipitation with anti-Flag agarose followed by elution with the Flag peptide and analyzed by Western blotting as described previously (18). Endogenous N-CoR was isolated from 293T cell lysates by immunoprecipitation with an anti-N-CoR antibody.

Growth inhibition and transcription reporter assays

For growth inhibition assay, $2x10^4$ Ba/F3 cells were incubated with various concentrations of TGF-β1 for 4 days. The cells were then counted and compared to unstimulated cells to determine the percent growth inhibition (45).

For the transcriptional reporter assay, a total of 2.5μg DNA were transfected into Hep3B cells. To examine TGFβ-induced transcriptional activation, Hep3B cells were transfected with 0.5 μg p3TPlux or pLUC800 (a luciferase reporter driven by the 800 bp natural promoter region of the PAI-1 gene) (49) and 2 μg SnoN, or with various

concentrations of Ski together with 0.5 μg p3TP-lux. Luciferase activity was measured 16 hrs after stimulation with 50 pM TGFβ1.

GST pull-down assay:

Recombinant wild-type or mutant Ski were expressed in and purified from *E.coli* as GST-fusion proteins. To test whether these GST-Ski proteins bind to Smad3, 1.5 μg GST-Ski immobilized on glutathione sepharose (Amersham Pharmacia) were blocked at 4°C for 30 minutes first with bacterial cell lysates followed by 0.2% BSA in GST binding buffer (20mM Hepes, 10% glycerol, 0.2mM EDTA, 1mM DTT, 100mM NaCl, 0.5% NP-40, 0.25mM KCl) (21). The immobilized GST-Ski was then incubated with 293T cell lysate expressing Flag-Smad3 for 1 hour in the GST binding buffer at 4°C. After extensive washes, Smad3 associated with GST-Ski was eluted with glutathione and detected by immunoblotting with anti-Flag MAb.

Soft-agar colony assay:

10^6 CEF cells were incubated with 10 μg DNA (Flag-Ski or Flag-SnoN in RCAS.BP) in the presence of 30 μg/ml polybrene. 6 hrs later, cells were shocked with 25% DMSO for 3.5 minutes. The transfected CEF were passaged for three weeks and the percentage of infected cells at this stage was above 70%. For the soft-agar colony assay, 4 ml of normal growth medium containing 0.66% agar was poured into a p50 dish to form the bottom layer (50). 10^4 cells were then suspended in 2 ml medium containing 0.44% agar and overlaid onto the hardened bottom layer. 4 ml fresh medium containing 0.44% agar was added to the dish every week. After three weeks' incubation, colonies were visualized by staining with

0.1 mg/ml MTT (MPBS thiazolyl Blue, Sigma) and scanned on an HP ScanJet J300C to visualize colonies.

Immunofluorescence:

To monitor the expression and localization of the introduced wt and mutant Ski or SnoN proteins, CEF cells growing on glass cover slips were fixed with 4% formaldehyde. The Flag-Ski or Flag-SnoN proteins were detected by staining with an anti-Flag MAb followed by Alexa fluro® 488-conjugated goat anti-mouse antibodies. Nuclei were detected by DAPI staining. Positively stained cells were scored for each transfected construct and used to calculate the efficiency of transfection/infection.

Electrophoresis mobility shift assay (EMSA):

Smad4, Ski/Smad4, SnoN/Smad4 or mutant Ski and SnoN were incubated as indicated with ^{32}P-labeled SBE probe ($2x10^4$ cpm) at room temperature for 20 minutes in binding buffer (25 mM Tris-HCl, pH7.5, 80 mM NaCl, 35 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 15 µg/ml poly dI-dC, 300 µg/ml BSA, and 2% NP-40) (51). For antibody supershift assays, 2 µg of specific antibodies were pre-incubated with the protein complex at 4°C for 30 minutes. The protein-DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel.

Pulse-chase assay:

293T cells growing in p50 dishes were washed with 2 ml DME lacking Met and Cys and then pulsed with 0.2 mCi/ml ^{35}S-express for 30 minutes. The cells were then chased in complete medium for various periods of time.

Results:

Mapping of Smad2, Smad3 and Smad4 binding sites in Ski and SnoN.

A series of deletion mutants of Ski were constructed to identify the Smad3 binding sites in Ski (left panel, Figure 1A). GST fusions of wild-type (WT) and mutant forms of Ski were immobilized on glutathione sepharose and used to precipitate Flag-tagged Smad3 from transfected cell lysates. Ski fragments containing either residues 1-241 (lane 5, Figure 1A) or 241-441 (lane 9) interacted with Smad3, suggesting that at least *in vitro*, there are two Smad3 binding sites in Ski. Since Ski(1-241) associated with Smad3 with a much higher affinity than Ski(241-441), the major binding site is located between residues 1 and 241. Further deletion analysis indicated that residues 16-23 are required for binding to Smad3 since fragment 16-728 bound to Smad3 to a similar extent as wt Ski but 24-728 interacted with Smad3 with a much lower affinity (Lane 1-3 and 8). Furthermore, mutations that changed the residues 16-23 to alanine either abolished (16-19A) or greatly impaired (20-23A) this interaction (lanes 6-7, Figure 1A).

The minor Smad3 binding site has been previously mapped to the region between residues 241 to 323 (17). Further analysis using deletions and point mutants of Ski showed that residues 316-319 are required for this weak interaction (data not shown). Mutant Ski fragment (241-441) with these residues changed to alanine no longer bound to Smad3 *in vitro* (lane 10, Figure 1A). This minor binding site does not appear to be sufficient to mediate the Ski-Smad interaction or repress TGFβ signaling *in vivo*. Mutant Ski or SnoN lacking the major Smad3 binding site but still retaining this minor binding site did not associate with Smad2 or Smad3 *in vivo* (Figure 1B and 1C), nor was this minor binding site alone able to

mediate repression of TGFβ-induced transcriptional activation (data not shown). Therefore, we focused on the major Smad3 binding site in all subsequent binding and functional assays.

To examine whether residues 16-19 in Ski are also required for binding to the Smad proteins *in vivo*, we mutated these residues to alanine (mS2/3, Figure 1B). Epitope-tagged WT or mS2/3 mutant Ski was co-transfected with HA-tagged Smads, and their interactions were analyzed by co-immunoprecipitation. As shown in Figure 1B, mutation of residues 16-19 disrupted the interaction of Ski with both Smad2 and Smad3, but not Smad4 *in vivo*, indicating that Smad2 and Smad3 bind to the same residues in Ski. This is not surprising, since Smad2 and Smad3 are 91% identical in amino acid sequence. Furthermore, these residues are well conserved in SnoN because mutation of corresponding residues in SnoN (residues 85-88, mS2/3) also disrupted binding of SnoN to Smad2 or Smad3 *in vivo* (Figure 1C).

The Smad4 binding site in Ski and SnoN was determined by mutagenesis and by structural studies. We have previously shown that H222 and E223 in Ski are important for binding to Smad4 (21). In the crystal structure of a Ski fragment bound to the Smad4 MH2 domain, both residues contribute to the extensive interface between Smad4 and Ski, with E223 generating an inter-molecular hydrogen bond and H222 stabilizing the Ski structure (44). In addition, several amino acid residues including W274, T271 and C272 also make direct contact with Smad4 (44). Mutation of these residues either individually (mS4W, Figure 1B) or in combination (mS4HE) abolished binding of Ski to Smad4 *in vivo* in a co-immunoprecipitation assay ((44) and Figure 1B). These residues are well conserved in SnoN (1). Mutation of H266, E267 or W318, greatly impaired binding of SnoN to Smad4 (Figure 1C).

Taken together, our results indicated that the R-Smads (Smad2 or Smad3) and Co-Smad (Smad4) interact with different regions in Ski or SnoN. Amino acid residues 16-19 in Ski or residues 85-88 in SnoN are required for interaction with Smad2 or Smad3, while multiple residues including H222-E223, W274 in Ski (or corresponding residues in SnoN) are critical for interaction with Smad4. Mutation of both regions completely abolished the association of Ski or SnoN with the Smad proteins *in vivo* (mS3S4W or mS3S4HE mutant, Figure 1B and 1C).

Interaction between Ski/SnoN and the Smad proteins is required for repression of TGFβ-induced transcriptional activation.

We have shown previously that Ski and SnoN can be recruited to the Smad-binding DNA elements (SBE) through interaction with the Smads and repress transcriptional activation of TGFβ target genes through multiple mechanisms (16-20). In an electrophoretic mobility shift assay, WT Ski or SnoN immunoprecipitated from cells co-transfected with Smad4 (Figure 2A) or Smad3 (data not shown) formed a complex with the SBE that could be supershifted with antibodies directed against the Smad protein or epitope tags of Ski or SnoN (lanes 2-4 and 8-10, Figure 2A) (16-20). No such complex was detected with mutant forms of Ski or SnoN defective in binding to the Smads (mS3S4HE mutants) (lanes 5-7 and 11-13, Figure 2A), indicating that the interaction of Ski or SnoN with the Smads is necessary for binding to the SBE.

When co-transfected with the TGFβ-responsive reporter constructs, WT Ski or SnoN readily repressed TGFβ-induced transcriptional activation of either the natural PAI-1 promoter or p3TPlux, but the mS3S4 mutants failed to do so (Figures 2B-2D). Interestingly, mutation of either the R-Smad or Smad4 binding site alone did not significantly affect the

repressive activity of SnoN (Figures 2C-D) or Ski (data not shown), suggesting that they can inactivate the activity of the Smad heteromeric complex through binding to either Smad proteins. This is consistent with our previous structural analysis showing that binding of Ski to either Smad2 or Smad4 results in disruption of the heteromeric Smad complex (44). Only after both binding sites are mutated is the repression by Ski or SnoN abolished.

To confirm that the lack of repression by the mS3S4 Ski or SnoN mutant is not due to a destabilization of the structure of Ski or SnoN by the mutation, we measured the half-lives of the mutant Ski and SnoN and examined their ability to interact with the co-repressor N-CoR. Because mis-folded proteins are usually degraded rapidly, we hypothesized that if mutation of Smad binding sites affects the folding of Ski or SnoN, the mS3S4 mutants should be less stable than the WT Ski or SnoN. Their interaction with N-CoR may also be affected by these mutations. However, in a pulse chase assay (Figure 3A), the mS3S4 mutant Ski and SnoN were as stable as their WT counterparts. In fact, the mutant SnoN was even more stable in the presence of Smad3 (Figure 3A) or TGFβ1 (data not shown) than WT SnoN, presumably because it does not bind to Smad3 and cannot be degraded as a result of Smad3-dependent poly-ubiquitination (38). In a co-immunoprecipitation assay, the mS3S4 mutant Ski or SnoN bound to endogenous N-CoR in a manner indistinguishable from WT Ski or SnoN (Figure 3B). However, while WT Ski or SnoN recruits N-CoR to the Smads (lanes 3 and 6, Figure 3C), the mS3S4 mutants failed to do so (lanes 2 and 5), most likely because of their inability to interact with the Smad proteins. Thus, the mS3S4 mutation did not disrupt the folding of Ski or SnoN. The lack of repressive activity of these mutants is due to their inability to be recruited to the TGFβ responsive promoter element by the Smads and a failure recruit N-CoR and the associated transcription repressors to the Smads. In addition, our

previous structural studies indicated that the mS3S4 mutant cannot disrupt the active conformation of a heteromeric Smad complex, nor does it block binding of Smad3 to the co-activator p300/CBP (44). Taken together, these results indicated that interaction between Ski/SnoN and the Smads is indeed necessary for the repression of TGFβ target gene expression.

Interaction of Ski and SnoN with the Smads is required to block TGF-β induced growth inhibition.

Ski and SnoN are oncoproteins that when overexpressed induce oncogenic transformation of chicken and quail embryo fibroblasts (9,52,53). Additionally, high levels of Ski or SnoN have been detected in many human cancer cells (1,10,11). We speculated that the ability of Ski and SnoN to interact with the Smad proteins and repress the growth inhibitory activity of TGFβ may contribute to the transforming activity of Ski and SnoN. In support of this hypothesis, overexpression of Ski or SnoN blocks TGFβ-induced growth inhibition (17,18). To confirm that this activity indeed requires the interaction with the Smads, the mutant forms of Ski or SnoN deficient in binding to the Smad proteins (mS2/3, mS4 and mS3S4) were introduced stably into the Ba/F3 pro-B cells and examined for their ability to block TGFβ-induced cell cycle arrest. We have shown previously that WT SnoN is rapidly degraded upon stimulation with TGFβ1 (17,18,54-56) and blocks TGFβ1-induced growth inhibition only modestly. We therefore used a truncated form of SnoN, SnoN(1-366), which can not be degraded by TGFβ and consequently is more potent than full-length SnoN in repression of TGFβ signaling (55). When expressed at similar levels (Figure 4A and data not shown), the mutant forms of Ski lacking one of the Smad binding sites retained the ability to block TGFβ-induced growth arrest, while mutants lacking both Smad binding sites

did not (Figure 4B). Similarly, the mS3S4 mutant SnoN lacking both Smad binding sites exhibited a markedly impaired ability to block TGFβ-induced cell cycle arrest (Figure 4C). This indicated that interaction of Ski and SnoN with the Smads is indeed required for antagonism of TGFβ-induced growth inhibition.

Interaction of Ski or SnoN with the Smads is critical for their transforming activity.

Previous studies have indicated that overexpression of Ski or SnoN in CEF induced anchorage-independent growth as measured by the soft-agar colony assay (9,52). To investigate whether this activity depends on the ability of Ski or SnoN to antagonize the growth inhibitory pathway of TGFβ, Flag-tagged wild type or mutant Ski or SnoN were cloned into a replication-competent avian retroviral vector, and the constructs were transfected into primary CEF. The extent of infection of the CEF and the expression of these Ski and SnoN proteins were monitored by immunofluorescent staining (Figure 5D) and by immunoblotting with anti-Flag. Three weeks after the initial transfection, approximately 70% of the cells were infected for each construct (data not shown). Both WT and mutant Ski or SnoN proteins were expressed at similar levels in the nucleus (Figures 5A and 5D and data not shown). In growth inhibition assays, WT but not mutant Ski blocks TGFβ-induced cell cycle arrest of CEF (data not shown), similar to that observed in Ba/F3 cells (Figure 4). In soft-agar colony assays, while CEF expressing WT Ski, SnoN or SnoN(1-366) grew readily in soft agar, those expressing the mutant Ski or SnoN proteins showed a greatly reduced ability to form soft-agar colonies (Figure 5A-5C), suggesting that the transforming activity of Ski or SnoN is dependent on their ability to interact with the Smad proteins. Interestingly, a Ski truncation 24-441 (human v-Ski equivalent) readily transformed CEF even though it only interacted with Smad4, but not the R-Smad (data not shown). Mutation of the Smad4

binding site from this molecule markedly impaired its transforming activity (data not shown), suggesting that the Ski-Smad4 interaction plays a crucial role in the transforming activity of Ski. Taken together, our results indicate that the ability of Ski and SnoN to interact with the Smad proteins and antagonize TGFβ-induced growth arrest is responsible for their transforming activity.

Discussion:

Ski and SnoN are expressed in most adult and embryonic cells, albeit at a low level. Under normal physiological conditions, elevation of Ski or SnoN expression only occurs during certain stages of embryogenesis and is under strict controls. When overexpressed, Ski and SnoN induce oncogenic transformation of CEF. In addition, transformation by Ski has also been reported in pigmented avian melanocytes and bone marrow-derived multipotent progenitor cells (11,14). High levels of Ski or SnoN expression were found in human melanoma, breast cancer, esophagus cancer and carcinoma of the vulva, stomach and lung (1,10,11,57). However, the mechanism by which Ski and SnoN induce transformation is unclear.

Recently we and others have shown that Ski and SnoN could interact with the Smad proteins and repress their ability to activate TGFβ signaling. To examine whether this is responsible for the transforming activity of Ski or SnoN, we first determined the amino acid residues in Ski or SnoN required for interaction with the Smads. Ski and SnoN were found to bind to the R-Smads, Smad2 and Smad3, and Co-Smad, Smad4, through different regions. The presence of two Smad binding sites suggests that Ski or SnoN can either interact with one of the Smad proteins individually (35,54,55) or bind to a heterodimer of R-Smad and Smad4, depending on the absence or presence of ligand and the status of the signaling pathway. Binding of Ski or SnoN to R-Smad or Smad4 individually may result in recruitment of Smad-associated cellular proteins to Ski or SnoN, allowing crosstalk with other intracellular signaling pathways or modulation of the activity or expression of Ski or SnoN. For example, binding of SnoN to the R-Smads independent of Smad4 results in recruitment of two ubiquitin ligases, Smurf2 and the anaphase promoting complex, leading to

the degradation of SnoN (54,55). Binding of Ski or SnoN to a heteromeric Smad complex, on the other hand, results in the disruption of such a functional complex since Ski and SnoN were found to compete with the R-Smads for binding to a common region in Smad4 (44). Although the disrupted Smad complexes remain bound to Ski, these complexes are not in an active conformation to interact with transcriptional co-activators such as CBP to activate TGFβ target genes (44). Based on this model, binding of Ski or SnoN to one of the Smad molecules, either a R-Smad or Smad4, is sufficient for the disruption of the Smad complex and subsequent repression of TGFβ signaling. Indeed, we found that mutation of one of the Smad binding sites in Ski or SnoN did not affect the ability of Ski or SnoN to repress the transactivation activity of the Smads significantly. Only when both binding sites are abolished was the repression of Smad function impaired. Consistent with this, although v-Ski lacks the R-Smad binding site due to a truncation of the first 27 amino acid residues from c-Ski, it still represses Smad function and induces potent transformation of CEF, probably because it can still interact with the Smad complex and inactivate it through binding to Smad4.

The residues in Ski that mediate Smad4 binding are well conserved in all Ski family members including v-Ski, c-Ski, c-SnoN and its isoforms (SnoN2, SnoI and SnoA), and the region required for R-Smad binding is present in all but v-Ski. A careful comparison of the amino acid sequence within the two Smad binding sites did not reveal any extensive homology between the two regions. These Smad binding sites do not shown any obvious similarity to the PPNK-containing motif found to mediate Smad2-binding in FAST1 and in the Milk family of proteins, or to the Smad-binding domains of SARA, TβRI or Smurf2 (58-62). Consistent with these observations, different domains or residues in Smad2 may

mediate binding to these proteins. For example, a basic-amino-acid-residue stretch in the L3 loop of Smad2 interacts with the activated TβRI, while the PY motif in the linker region of Smad2 recognizes the WW domain in Smurf2 (54). Thus, Smad2 and Smad3 may recognize multiple sequence motifs through different amino acid residues. Interestingly, the R-Smad binding site is not 100% conserved between Ski and SnoN. This partial difference may contribute to the different affinity of Ski and SnoN for the BMP Smads (17,18). A thorough understanding of the Ski-Smads and SnoN-Smads interactions will require a detailed analysis of the three-dimensional crystal structures of these complexes.

Because Smad proteins are important tumor suppressors, we hypothesized that the ability of Ski and SnoN to inactivate Smad function may be responsible for their transforming activity. In this study, we employed CEF as a model system to investigate the transforming activity of Ski and SnoN because Ski was originally identified in CEF as a viral oncogene by virtue of its ability to transform CEF. Unlike many mammalian fibroblast cell lines that proliferate in response to TGFβ, CEF, like many epithelial cells, undergo growth arrest in the presence of TGFβ (data not shown). Thus, overexpression of Ski or SnoN blocks TGFβ-induced growth arrest (data not shown), and this may be responsible for the transformation of CEF. Indeed, we have shown here that the interaction of Ski/SnoN with Smad proteins is required for the antagonism of TGFβ signaling and more importantly, for their transforming activity. Mutant Ski and SnoN lacking the Smad binding sites are defective in repression of TGFβ-induced cell cycle arrest and fail to induce anchorage-independent growth of CEF. Extrapolating from this, the high level expression of Ski or SnoN in some human cancer cells may be responsible for the resistance of these cancer cells

to TGFβ-induced growth arrest, a key step in the malignant progression of mammalian tumor cells.

TGFβ signaling pathways are considered both a tumor suppressor pathway and a promoter of tumor progression and invasion. TGFβ1, TGFβ receptors and the Smad proteins are expressed in virtually all tissue and cell types. Activation of TGFβ1 and TGFβ signaling *in vivo* can be regulated at both the level of extracellular ligand activation and intracellular signal transduction. These highly regulated processes regulate the differentiation or proliferation state of a given cell type or tissue. In normal cells and at early stages of tumorigenesis, activation of TGFβ and Smad proteins inhibit cell growth. Perturbation of this growth inhibitory pathway by activation or overexpression of oncogenes such as *ski* and *snoN* results in a diminished growth inhibitory response, leading to rapid tumor growth and clonal expansion and permits the accumulation of further mutations and tumor progression. As tumor cells lose their ability to be inhibited by TGFβ and progress to a more malignant stage, stimulation by TGFβ causes these cells to undergo epithelial to mesenchymal transdifferentiation leading to increased tumor metastasis and invasion. Thus the activity of Ski and SnoN may function to promote the switch of the tumor cell responses to TGFβ from growth inhibition to accelerated malignant progression.

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Figure legends:

Figure 1. Mapping of Smad2, Smad3 and Smad4 binding sites in SnoN and Ski.

(A). Ski contains a major and a minor Smad3 binding site. Left panel: Diagram of the Ski mutants. Right panel: GST-pull down assay. Wild-type (WT) or mutant GST-Ski proteins immobilized on glutathione sepharose were incubated with cell lysate from 293T cells transfected with Flag-Smad3. After extensive washes, the Ski-bound Flag-Smad3 was eluted with glutathione and visualized by western blotting with anti-Flag (upper panel). GST-Ski proteins were visualized by Coomassie Blue staining as a control for GST-Ski input (bottom panel).

(B and C). Interaction of Ski or SnoN with the Smad proteins *in vivo.* Left panels: Ski or SnoN mutants. Right panels: co-immunoprecipitation assays. Flag-tagged WT and mutant Ski (**B**) or SnoN (**C**) were co-transfected into 293T cells together with HA-tagged Smads and isolated by immunoprecipitation with anti-Flag. The Ski or SnoN-bound Smads were visualized by western blotting of the immunoprecipitates with anti-HA. Cell lysates were blotted directly with anti-HA as a control for expression of the HA-Smads.

Figure 2. Interaction of Ski or SnoN with the Smad proteins is required for repression of transcriptional activation.

(A). Interaction with the Smad proteins is required for the binding of Ski or SnoN to SBE. Smad4 in complex with the Flag-tagged Ski or SnoN was isolated from transfected 293T cells by immunoprecipitation with anti-Flag followed by elution with the Flag peptide (lanes 2-13). As a positive control, recombinant Smad4 was isolated from *E.coli* as a GST-fusion protein (lane 1). These protein complexes were then incubated with ^{32}P-labeled SBE

oligo. The resulted DNA-protein complexes were resolved by 4% non-denaturing polyacrylamide gel electrophoresis. Proteins used in the EMSA reactions: purified Smad4: 0.2µg; WT Ski or SnoN-bound Smad4: 0.2µg; lane 1: GST-Smad4; lane 2-4: Flag-SnoN/Smad4 complex purified from co-transfected 293T cells; lane 5-7: Flag-SnoN mS3S4/Smad4 complex; lane 8-10: Flag-Ski/Smad4 complex; lane 11-13: Flag-Ski mS3S4/Smad4 complex.

(B-D). Repression of transcription by the Ski and SnoN proteins. Hep3B cells were transfected with two different concentrations of *ski* together with 0.5µg p3TP-lux (**B**) or with 2µg WT or mutant *snoN* and 0.5µg pLUC800 (**C**) or p3TP-lux (**D**). Luciferase activity was measured 48 hrs later.

Figure 3. (**A**) **Stability of the WT and mutant Ski or SnoN proteins**. Flag-tagged WT or mutant Ski or SnoN were transfected into 293T cells either alone or together with Smad3 and isolated by immunoprecipitation with anti-Flag agarose. The pulse-chase assay was carried out two days after transfection as described in Materials and Methods. Wild-type proteins are on the left panel and the mutant proteins are on the right.

(**B) Mutant Ski or SnoN proteins defective in Smad binding can still interact with N-CoR**. Flag-tagged WT or mutant Ski or SnoN were transfected into 293T cells. Endogenous N-CoR was isolated by immunoprecipitation with anti-N-CoR antibody (middle panel). The Ski or SnoN proteins associated with N-CoR was visualized by western blotting of the immunoprecipitates with anti-Flag (upper panel). The expression level of Ski or SnoN was measured by western blotting of the cell lysates with anti-Flag (bottom panel).

(C) Mutant Ski or SnoN defective in Smad binding cannot recruit N-CoR to the Smads. HA-Smad4 was transfected into 293T cells either alone (lanes 1 and 4) or together with WT

(lanes 3 and 6) or mutant (lanes 2 and 5) SnoN or Ski proteins and isolated by immunoprecipitation with anti-HA. Endogenous N-CoR associated with HA-Smad4 was detected by western blotting with anti-N-CoR (top panel). As controls, the anti-HA immunoprecipites were blotted with anti-Flag for the associated SnoN or Ski proteins (second panel from the top) or with anti-HA for Smad4 (third panel). Total cell lysates were blotted with anti-N-CoR or anti-Flag to control for the expression of N-CoR and SnoN or Ski proteins (bottom two panels).

Figure 4. Interaction of Ski and SnoN with the Smads is critical for blocking TGFβ-induced growth inhibition.

(**A**). Uninfected Ba/F3 cells (lane 1) or Ba/F3 cells stably expressing wild type or mutant Flag-Ski (lanes 4 and 5) or Flag-SnoN(1-366) (lanes 2 and 3) were generated by retroviral infection. The expression of the Ski and SnoN proteins was detected by immunoprecipitation with anti-Flag agarose followed by western blotting with anti-Flag. Uninfected Ba/F3 cells and stable cell lines expressing Ski (**B**) or SnoN(1--366) (**C**) were incubated for 4 days with various concentrations of TGF-β1 as indicated. The growth of the cells was quantified by cell counting and compared to that of unstimulated cells.

Figure 5. Interaction of Ski with the Smads is critical for the transforming activity of Ski and SnoN.

Flag-tagged WT or mutant Ski and SnoN were introduced into CEF by a transfection. Three weeks after the initial transfection, a soft-agar colony assay was performed as described in Materials and Methods. The soft-agar plates were stained with MTT and scanned, and the Ski plates are shown in (**A**). The expression levels of the WT or mutant Ski in these cells

were determined by western blotting with anti-Flag, shown on the right. (**B**) Microscopic view of representative regions from the MTT-stained soft-agar plates expressing various SnoN constructs. The number of soft-agar colonies in a representative 2.5cm^2 area was quantified and is summarized in (**C**). (**D**) Flag-Ski and SnoN expressed in CEF were visualized by immunofluorescent staining with anti-Flag (left panels) to control for the localization of Ski or SnoN. DAPI staining was performed to show the nuclei (right panels).

Figure 1, He et al.

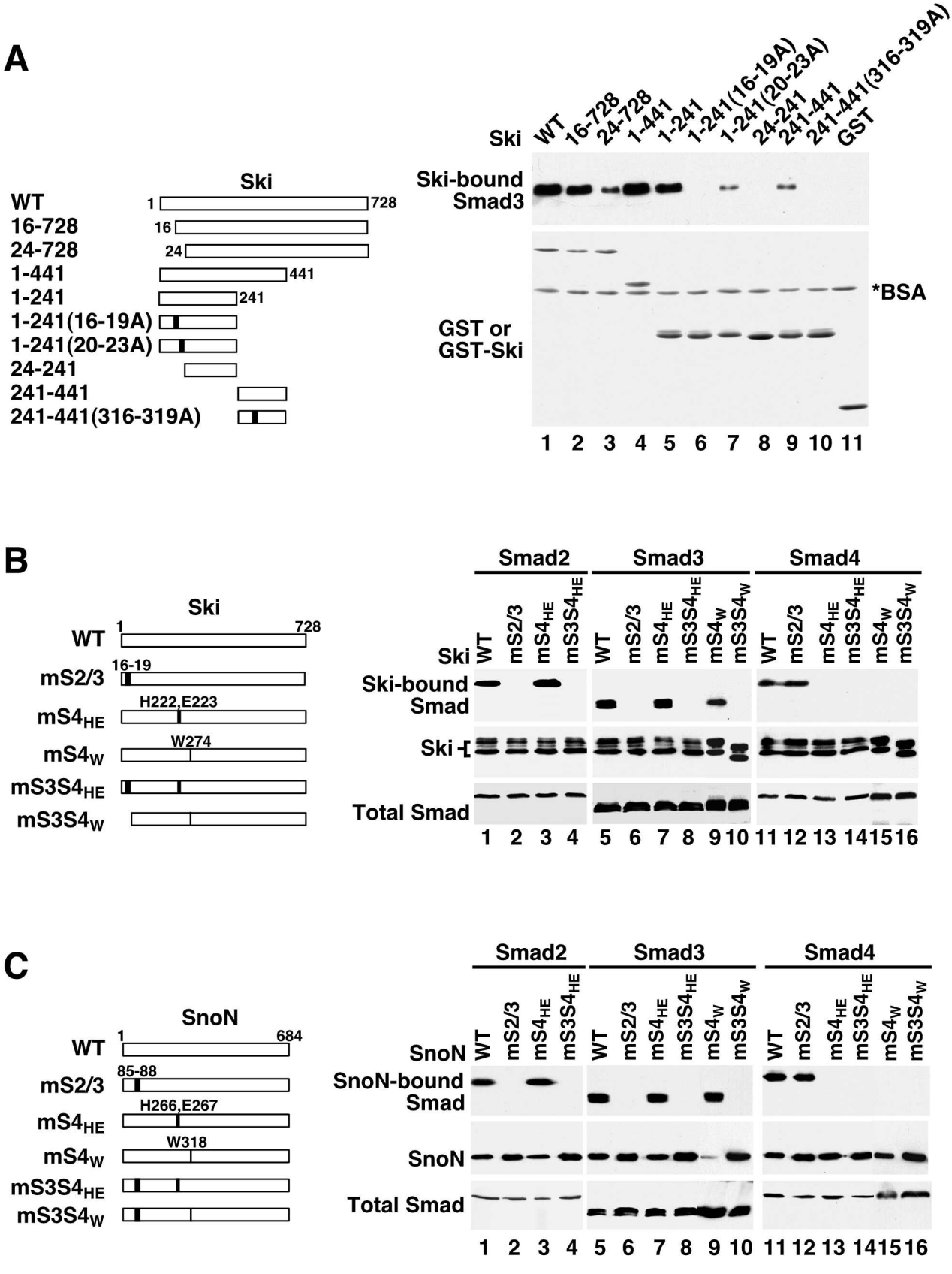

Figure 2, He et al.

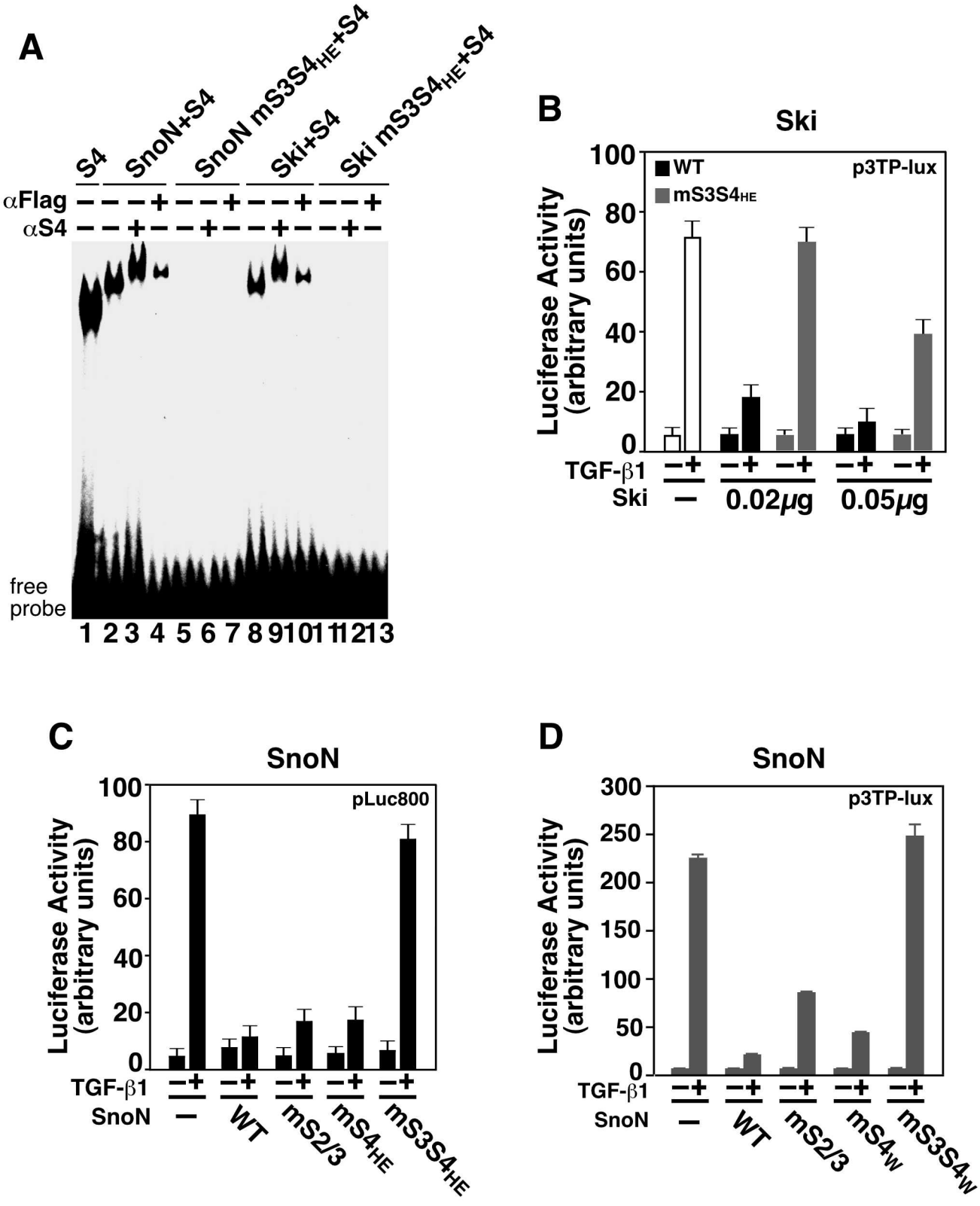

Figure 3, He et al.

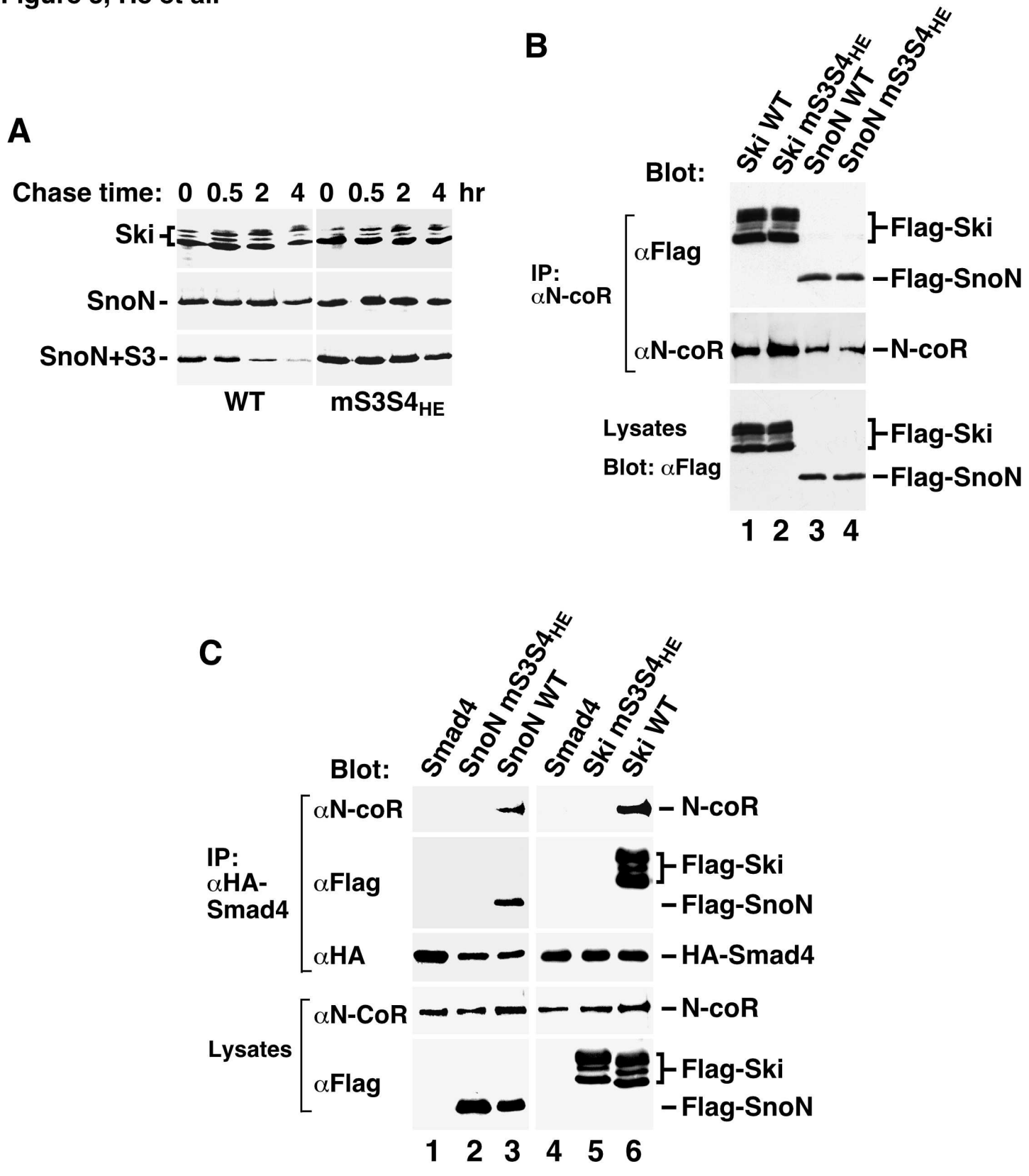

Figure 4, He et al.

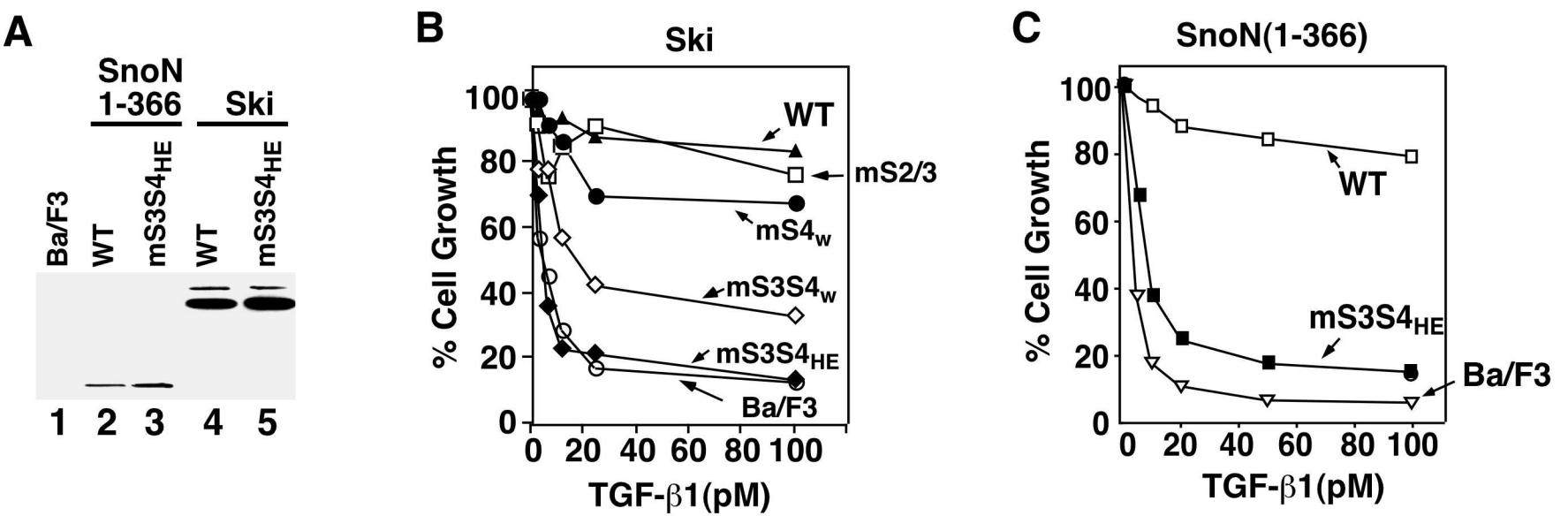

Figure 5, He et al.