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Transforming Growth Factor-β Suppresses the Ability of Ski to Inhibit Tumor Metastasis by Inducing Its Degradation

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Abstract

c-Ski is an important corepressor of transforming growth factor-β (TGF-β) signaling through its ability to bind to and repress the activity of the Smad proteins. It was initially identified as an oncogene that promotes anchorage-independent growth of chicken and quail embryo fibroblasts when overexpressed. Although increased Ski expression is detected in many human cancer cells, the roles of Ski in mammalian carcinogenesis have yet to be defined. Here, we report that reducing Ski expression in breast and lung cancer cells does not affect tumor growth but enhances tumor metastasis *in vivo*. Thus, in these cells, Ski plays an antitumorigenic role. We also showed that TGF-β, a cytokine that is often highly expressed in metastatic tumors, induces Ski degradation through the ubiquitin-dependent proteasome in malignant human cancer cells. On TGF-β treatment, the E3 ubiquitin ligase Arkadia mediates degradation of Ski in a Smad-dependent manner. Although Arkadia interacts with Ski in the absence of TGF-β, binding of phosphorylated Smad2 or Smad3 to Ski is required to induce efficient degradation of Ski by Arkadia. Our results suggest that the ability of TGF-β to induce degradation of Ski could be an additional mechanism contributing to its protumorigenic activity. [Cancer Res 2008;68(9):3277–85]

Introduction

Ski was identified as the transforming protein (v-Ski) of the avian Sloan-Kettering virus (1). The cellular homologue of *v-ski, c-ski*, was later cloned based on sequence homologies (2). The oncogenic activity of Ski is primarily defined by its ability to promote anchorage-independent growth of chicken and quail embryo fibroblast when overexpressed (3). Consistent with this, Ski has been found to be expressed at high levels in human cancer cells derived from melanoma, esophageal cancer, colorectal cancer, pancreatic cancer, and leukemia (4–8). However, Ski may also contain an antitumorigenic activity. $Ski^{+/-}$ mice were found to display an increased susceptibility to chemical-induced tumorigenesis (9). The human *ski* gene is located at chromosome 1p36, a potential tumor suppressor locus that is frequently deleted in neuroblastoma and melanoma (10–12). Clearly, the roles of Ski in mammalian tumorigenesis are complex, and more studies are needed to define the functions of Ski. We have recently reported that SnoN exerts both pro-oncogenic and anti-oncogenic activities at different stages of tumorigenesis (13). Whether Ski has similar activities at different stages of mammalian carcinogenesis or exerts opposite effects in cancer cells of various origin has yet to be determined.

Ski and SnoN are important negative regulators of transforming growth factor-β (TGF-β) signaling (14). TGF-β family of cytokines plays important roles in the regulation of mammalian cell growth, differentiation, and cancer (15–19). In normal epithelial cells or during early stages of carcinogenesis, TGF-β acts as a tumor suppressor by promoting cell cycle arrest or apoptosis and through maintaining tissue homeostasis. These growth-inhibitory activities are often inactivated during malignant progression. At later stages of tumorigenesis, TGF-β promotes tumor invasiveness and metastasis through its ability to induce epithelial to mesenchymal transdifferentiation (EMT) and to modulate the extracellular stromal microenvironment in favor of tumor growth.

Smad proteins are important mediators of TGF-β signaling (20, 21). On ligand binding, active TGF-β receptor (TGF-βR) complex phosphorylates the receptor-activated Smad proteins (R-Smad) Smad2 and Smad3. Phosphorylated R-Smads then form heteromeric complexes with a common mediator Smad, Smad4, and translocate into the nucleus. In collaboration with other transcription factors, the heteromeric Smad complexes bind to promoter DNA and activate or repress transcription of TGF-β target genes. Smad complexes are subjected to positive and negative regulation through association with cellular partners. In particular, we and others have shown that Ski interacts with Smad2, Smad3, and Smad4 and represses their ability to activate TGF-β–responsive genes through disrupting the functional heteromeric Smad complexes, recruiting a transcriptional corepressor complex and preventing the binding of transcriptional coactivators (14). As a negative regulator of TGF-β signaling, it is conceivable that Ski may also possess both tumor-promoting and tumor-suppressive activities at different stages of tumorigenesis.

Ski is expressed in almost all adult and embryonic tissues but at low levels (1, 2, 22–24). Up-regulation of Ski expression occurs during certain stages of embryonic development and later in some human cancers. *In vivo* studies using *Xenopus*, zebrafish, or mice have shown that Ski plays critical roles during development of neuronal, craniofacial, and muscle lineages, possibly through modulating bone morphogenetic protein signaling (25–27). The activity and expression of Ski can be regulated at the level of transcriptional activation, posttranslational modification, protein stability, as well as intracellular localization. In melanoma, Ski can be up-regulated at the transcriptional level through yet to be identified mechanism (28). Although Ski has been identified as a nuclear protein, in invasive melanoma Ski localizes also in the cytoplasm. How this may affect its function in melanoma is still unclear (4).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Ski is also regulated by ubiquitin-dependent proteasome. During renal fibrosis induced by obstructive injury, Ski degradation is enhanced (29). Macdonald et al. (30) have shown that Ski stability can be regulated by the ubiquitin-conjugating enzyme Cdc34 in a cell cycle–dependent manner. The steady-state level of Ski may be additionally regulated by phosphorylation, which prevents Ski degradation during mitosis (31). Finally, two recent studies have reported that overexpression of Arkadia, an E3 ligase, induces the degradation of SnoN and Ski (32, 33).

It is well known that TGF-β expression is often up-regulated in metastatic tumors (16, 18). TGF-β can induce efficient ubiquitination and degradation of SnoN by multiple ubiquitination pathways and has been reported to induce the degradation of a transfected Ski in Mink lung epithelial cells (34–37). However, unlike the case of SnoN, degradation of Ski is not readily observed in all cell types (38). Although Ski can be degraded by overexpression of Arkadia (33), whether this pathway, when present at physiologic concentrations, is responsible for TGF-β–induced degradation of Ski is not clear. More importantly, the cellular context under which this degradation occurs has not been defined.

In this study, we investigated the function of Ski in tumorigenesis by down-regulating its expression in A549 lung cancer cells and MDA-MB-231 breast cancer cells. We report that down-regulation of Ski expression does not suppress tumor growth but enhances tumor metastasis *in vivo*. We also found that TGF-β induces efficient degradation of Ski through the ubiquitin-dependent proteasome pathway. Phosphorylated Smad2 and Smad3 cooperate with Arkadia to induce efficient degradation of Ski. Finally, as TGF-β is highly expressed in metastatic tumors, our result that TGF-β induces degradation of Ski may suggest an additional mechanism supporting the prometastatic role of TGF-β.

Materials and Methods

Cell culture. Melanoma cells were maintained as described previously (39). 293T cells, A549 lung adenocarcinoma cells, A375 melanoma cells, MCF7, MDA-MB-231, MDA-MB-468, MDA-MB-435, BT474, T47D, ZR75B, and BT549 human breast cancer cells, and MCF-10A mammary epithelial cells were maintained as described previously (13). These breast cancer cell lines were generous gifts of M.J. Bissell (Life Sciences Division, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA).

Assays for TGF-β responses. A549 and MDA-MB-231 cells were transfected with the p3TP-lux reporter constructs. Twenty-four hours after transfection, cells were serum starved for 8 h and luciferase activity was measured 16 h after stimulation with 50 pmol/L TGF-β1. For the growth inhibition assay, 3.5×10^4 MDA-MB-231 or A549 cells were cultured for 4 d with the indicated concentrations of TGF-β1. The growth of cells was determined by cell counting.

Transformation and tumor growth assays. Soft agar assays to analyze anchorage-independent growth were performed as described previously (13). To measure tumorigenicity *in vivo* in nude mice, female athymic BALB/c mice (Charles River Laboratories) at 4 to 6 wk of age were injected s.c. with various cancer cell lines and tumor growth was monitored as described earlier (13).

***In vivo* metastasis assays.** MDA-MB-231 cells (1×10^5 in 0.1 mL PBS/mouse) were injected into the left cardiac ventricle of 4-wk-old female nude mice. Animals were monitored for paraplegia and the body weight of each mouse was measured weekly. After 4 wk, mice were sacrificed and the lungs were fixed in Bouin's solution. Metastatic nodules were counted at the lung surface under a dissecting microscope. Bone metastasis in tibia was determined as previously described (13).

To detect paraplegia as a measure of skeletal muscular function due to bone metastasis, a wire hang test was performed as previously described (13). The results were indicated as mean latency to fall from the lid in seconds ± SE.

To examine metastasis of A549 lung cancer cells, 2×10^6 cells in 0.1 mL PBS were injected into the lateral tail veins of 4-wk-old female nude mice. After 3 mo, the mice were sacrificed and the number of metastasis nodules was determined on lungs fixed in Bouin's solution (13).

Cell lysis, immunoprecipitation, and immunoblotting. Cell lysis, immunoprecipitation, and Western blotting were performed as previously described (13). For inhibition of proteasome degradation, cells were pretreated with 50 μmol/L MG132. For TGF-β–mediated degradation of Ski, 100 pmol/L TGF-β1 was used. In some experiments, the cells were pretreated for 1 h with either 10 μmol/L SB-431542, 10 μmol/L SP600125, 5 μmol/L SB-20358, or 10 μmol/L Y-27632.

Results

Down-regulation of Ski expression enhances tumor metastasis *in vivo*. To investigate the role of Ski in malignant transformation, we stably introduced a short hairpin RNA (shRNA) directed against human Ski into MDA-MB-231 and A549 cells to reduce its expression. MDA-MB-231 is a metastatic human breast cancer cell line that expresses high levels of Ski and SnoN (13). A549 human lung adenocarcinoma cells express high levels of SnoN but low levels of Ski (13). Both cell lines are resistant to the cytostatic effect of TGF-β. pSUPER vector expressing *ski* shRNA was introduced into these cells by transfection together with a plasmid expressing a puromycin resistance gene. A significant reduction of Ski expression was detected in several stable puromycin-resistant clones (Fig. 1*A*).

Because Ski is a negative regulator of TGF-β signaling, we first tested whether down-regulation of Ski expression enhanced TGF-β–induced transcription. Parental and shSki cells were transfected with the p3TP-lux luciferase reporter followed by treatment with TGF-β. Whereas reducing Ski expression did not affect basal reporter activity, TGF-β–induced transcription was enhanced in both MDA-MB-231 and A549 shSki cells. Thus, reducing Ski expression enhanced TGF-β signaling.

Both A549 and MDA-MB-231 cells are resistant to growth inhibition by TGF-β, possibly due to high levels of SnoN and Ski expression. Interestingly, reducing Ski expression in these cells was not sufficient to elicit a growth-inhibitory response to TGF-β (Fig. 1*C*), most likely because of the presence of high levels of SnoN and the relative expression level of Ski in these cell lines. The low level of Ski expression in A549 cells may account for this lack of effect, and the presence of high levels of SnoN in these cells probably contributes to the inhibition of TGF-β signaling. Indeed, when both Ski and SnoN were knocked down, the growth of A549 cells was inhibited effectively by TGF-β and a partial growth arrest was observed in MDA-MB-231 cells. Thus, it seems that Ski expression in tumor cells contributes to their resistance to TGF-β–induced growth arrest.

In a soft agar assay to measure the transforming activity of these cells, reducing Ski expression in MDA-MB-231 and A549 cells had no effect on anchorage-independent growth (Fig. 1*D*). Consistent with this, Ski knockdown did not affect tumor growth *in vivo* in nude mice (data not shown). In contrast, reducing SnoN expression fully blocked soft agar colony formation (13). Thus, unlike the related SnoN and in contrast to its transforming activity in chicken embryo fibroblasts, Ski does not seem to affect mitogenic transformation of human breast and lung cancer cells.

Given that Ski did not affect tumor growth in our assays, we next compared the ability of MDA-MB-231 parental and shSki cells to

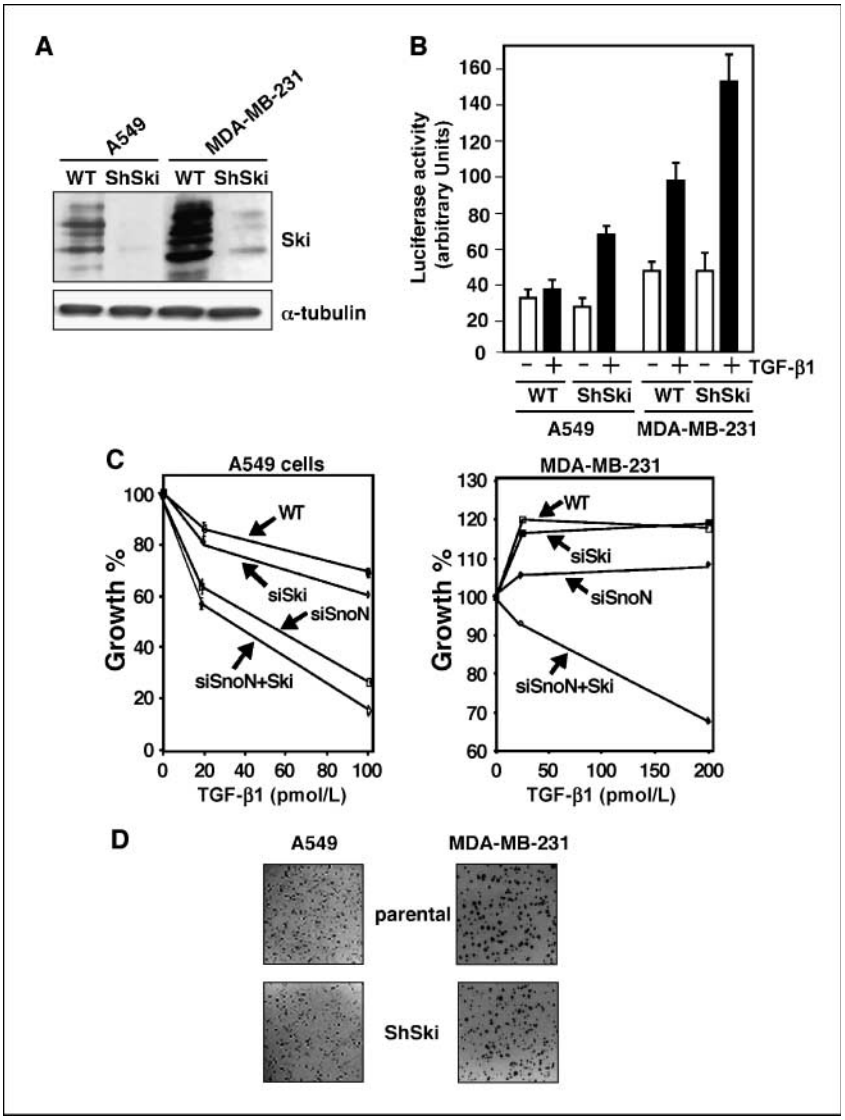

Figure 1. Ski does not affect mitogenic transformation of human cancer cells. *A,* reduction of Ski expression in A549 lung cancer cells and MDA-MB-231 breast cancer cells by shRNA. Stable cell lines expressing the shRNA for human *Ski* were generated as described in Materials and Methods. Ski expression in the representative stable clones was assessed by immunoprecipitation and Western blotting with anti-Ski. Whole-cell extracts were blotted with anti-α-tubulin as a loading control. *B,* down-regulation of Ski expression enhanced TGF-β–induced transcription. Parental cell lines and their shSki derivatives were transfected with p3TP-lux and serum starved. Luciferase assays were performed 16 h after stimulation with 50 pmol/L TGF-β1. *C,* growth inhibition assay. Parental and shRNA-expressing cells were treated with increasing concentrations of TGF-β1 and cultured for 4 d. The growth of cells was determined by cell counting and expressed as a percentage of the number of unstimulated cells. *D,* reducing Ski expression had no effect on anchorage-independent growth of A549 and MDA-MB-231 cells. Parental shSki cells were subjected to a soft agar colony assay as described in Materials and Methods. Representative images after staining with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide are shown.

induce secondary lung and bone metastases in an *in vivo* metastasis assay. As shown in Supplementary Fig. S1, although both parental and shSki cells induced bone and lung metastasis, mice inoculated with shSki cells displayed a moderate but significant and reproducible increase in lung metastasis. These mice also showed a 2-fold increase in bone metastasis (Fig. 2*A*) as well as a more severe paraplegia, as indicated by the reduced latency to fall down from a wire lid in a wire hang test (Fig. 2*B*). Thus, down-regulation of Ski enhances the metastatic potential of MDA-MB-231 breast cancer cells. Similar to what was observed with MDA-MB-231 cells, A549 lung cancer cells with reduced levels of Ski displayed a 2-fold increase in metastatic potential over the parental cells (Fig. 2*C*).

The ability of Ski to inhibit tumor metastasis was further substantiated by microarray analysis. Using the Affymetrix U133(A+B) arrays to compare the transcriptome of parental MDA-MB-231 and shSki cells, we identified among the group of genes whose expressions were altered by Ski knockdown a set of genes known to be involved in regulation of EMT and metastasis or identified as prognostic markers of metastasis. A subset of these genes is shown in Supplementary Table S1. The differential expression of some genes was further confirmed by reverse transcription-PCR (RT-PCR; Fig. 2*D*). Consistent with the observed increase in metastasis, shSki cells showed up-regulation of many metastasis-promoting genes, including *AGR2, HMGA2*, and *SLUG*, and down-regulation of genes inhibitory to metastasis, including *KISS-1* and *FHL1*. Some of these genes are also regulated by the Smad proteins and TGF-β signaling. For examples, among the genes that are up-regulated in shSki cells, *HMGA2* and *SLUG* can be induced by TGF-β in a Smad-dependent manner (40, 41). Other genes, such as *FHL1*, a tumor suppressor gene that acts downstream of Src and Cas to block tumor migration, is down-regulated in shSki cells but has not yet been identified as a TGF-β–responsive gene. These results suggest that Ski may regulate tumor metastasis *in vivo* through both TGF-β–dependent and TGF-β–independent pathways.

Taken together, these observations indicate that Ski inhibits tumor metastasis *in vivo*.

TGF-β–induced degradation of Ski. Ski is located at chromosome 1p36, a locus frequently deleted in melanoma (10, 12). This observation, together with the ability of Ski to inhibit tumor metastasis, would predict that its expression is reduced in

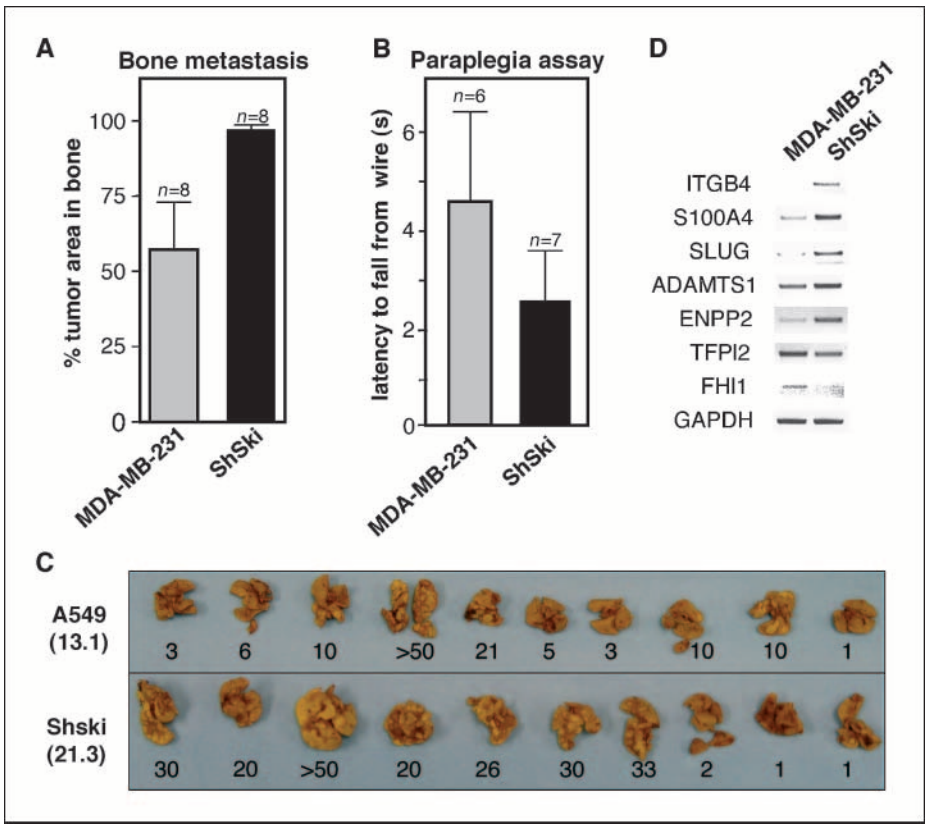

Figure 2. Down-regulation of Ski expression enhances tumor metastasis *in vivo*. *A,* abrogation of Ski expression increased bone metastasis. Parental or shSki-expressing MDA-MB-231 cells were subjected to *in vivo* metastasis assay as described in Materials and Methods. The bone metastatic potential is indicated by the histomorphometric measurement of tumor area/burden (in percentage) in the cancerous regions of the right proximal tibia. *Columns,* mean from eight parental cell–injected mice and eight shSki cell–injected mice; *bars,* SE. *B,* paraplegia assay. A wire hang test was performed as described in Materials and Methods. *Columns,* mean from six parental cell–injected mice and seven shSki cell–injected mice; *bars,* SE. *C,* A549 lung cancer metastasis assay. Parental or shSki-expressing A549 cells were injected into the tail veins of 4-wk-old female nude mice as described in Materials and Methods. After 3 mo, the average numbers of tumor nodules present in the lungs from these mice were counted and indicated in parenthesis. *D,* RT-PCR analysis of the transcription of genes whose expressions were altered by Ski knockdown in MDA-MB-231 cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was included as a control.

metastatic cancer cells. We therefore examined expression of Ski in a panel of metastatic melanoma cells and a variety of breast cancer cell lines. Surprisingly, Ski was expressed in all metastatic melanoma cell lines at reasonably high levels as well as in all breast cancer cell lines (Fig. 3*A*). No major difference in the steady-state level of Ski was detected between nonmetastatic breast cancer cell lines (MCF7, BT474, ZR75B, and T47D) and highly invasive, metastatic breast cancer lines (MDA-MB-231, BT549, and MDA-MB-435; Fig. 3*A*). This raised the important question of whether Ski expression in these cell lines is subjected to dynamic regulation by cytokines or factors known to be active during metastasis.

Because TGF-β is often up-regulated in metastatic tumor cells and has been reported to induce Ski degradation under some circumstances, we next examined the level of Ski expression when various human cancer cell lines were treated with TGF-β1. As shown in Fig. 3*B*, Ski protein level in MDA-MB-231 cells was markedly reduced on TGF-β treatment, with a maximal effect after 40 min of TGF-β treatment, and remained barely detectable 4 h later. These results showed that TGF-β induced a dramatic reduction of Ski protein in MDA-MB-231. To assess how general this regulation is, we checked for the regulation of Ski expression by TGF-β in a panel of human breast cancer and melanoma cell lines. TGF-β induced a moderate reduction of Ski expression in several breast cancer cell lines, including BT474, MDA-MB-231, and MDA-MB-435, but not in T47D and ZR75B cells (Fig. 3*C*), which do not express the TGF-βRII, or in MDA-MB-468 cells (data not show), which carry a chromosomal deletion encompassing the entire Smad4 locus (42). Similar data were obtained in melanoma cell lines (Supplementary Fig. S2*A*). Altogether, these data showed that TGF-β can reduce the level of Ski in various metastatic cancer cell lines. Interestingly, although melanoma cells are resistant to the TGF-β–induced growth inhibition, mutations of the TGF-βRs or the Smad proteins have not been reported. Thus, an active TGF-β/Smad signaling pathway may be present in these melanoma cells to regulate the expression of Ski.

We next evaluated whether TGF-β affected the transcription or degradation of Ski. MDA-MB-231 cells were treated with TGF-β, and Ski transcription was monitored by semiquantitative RT-PCR and Ski degradation was measured by Western blotting after treatment with a proteasome inhibitor. TGF-β did not affect Ski transcription (Supplementary Fig. S2*B*) but induced degradation of the Ski protein because treatment of cells with MG132 abrogated TGF-β–induced down-regulation of Ski (Fig. 3*D*). Thus, TGF-β induces degradation of Ski through the proteasome pathway.

Degradation of proteins by the proteasome requires prior polyubiquitination. To determine whether Ski is polyubiquitinated in response to TGF-β, Flag-Ski was transfected into 293T cells together with or without the constitutively active ALK5 and isolated by immunoprecipitation. Ubiquitination of Ski was detected by Western blotting with anti-ubiquitin antibodies. As shown in Supplementary Fig. S2*C*, activation of TGF-β signaling readily induced polyubiquitination of Ski. Interestingly, when the Smad-binding sites were mutated in Ski, this polyubiquitination was eliminated (Supplementary Fig. S2*C*), suggesting that the Smad pathway may be involved in Ski degradation.

TGF-β–induced degradation of Ski through the Smad pathway. TGF-β activates both Smad-dependent and Smad-independent pathways, such as c-Jun NH_2-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK), and RhoA pathways. To determine which of these pathways is activated by TGF-β to down-regulate Ski expression, specific pharmacologic inhibitors of JNK (SP600125), p38MAPK (SB-20358), and ROCK

(Y-27632) were used. As a positive control, SB-431542, the TGF-βRI inhibitor, was included. MDA-MB-231 cells were pretreated for 1 h with various inhibitors at concentrations shown to fully inhibit their target pathways without significant nonspecific activity followed by treatment with or without TGF-β for 40 min. In the absence of TGF-β, Ski expression was slightly stabilized by SB-431542 (Fig. 4*A*), suggesting that inhibition of autocrine TGF-β signaling may increase Ski stability. In the presence of TGF-β, degradation of Ski was abrogated efficiently by SB-431542 (Fig. 4*A*). In contrast, inhibitors of JNK, p38MAPK, and ROCK did not affect the level of Ski either in the absence or in the presence of TGF-β (Fig. 4*A*). This suggests that Ski degradation by TGF-β is most likely mediated by the canonical Smad pathway.

To further confirm this point, pool of small interfering RNA (siRNA) duplex directed against Smad2 and Smad3 was transfected into MDA-MB-231 cells before the TGF-β treatment. Individual knockdown of Smad2 and Smad3 did not affect TGF-β–induced degradation of Ski (Fig. 4*B*). However, when both Smad2 and Smad3 were reduced by concomitant siRNA transfection, Ski degradation in response to TGF-β was strongly impaired (Fig. 4*B*). These indicate that Smad2 and Smad3 act in a complementary manner to degrade Ski. Altogether, these experiments show that activation of Smad2 or Smad3 by TGF-β is necessary to induce the proteasome degradation of Ski.

Arkadia is required for TGF-β–induced degradation of Ski. Several E3 ligases, including Smurf1, Smurf2, Arkadia, and the APC/Cdh1 complex, have been implicated in the regulation of TGF-β signaling. To determine which E3 ligase is responsible for the degradation of Ski, we examined the ability of overexpressed Smurf1, Smurf2, Arkadia, and APC/Cdh1 to induce Ski degradation in 293T cells. As shown in Fig. 5*A*, overexpression of Smurf1, Cdh1, and Arkadia strongly reduced the level of Ski, whereas Smurf2 did not affect Ski stability. This indicates that Smurf1, Arkadia, and the APC/Cdh1 complex are potential candidates to induce Ski degradation. To address more directly which E3 ligase is recruited on TGF-β stimulation to mediate Ski degradation, various siRNA duplexes targeting Smurf1, Smurf2, Cdh1, or Arkadia were introduced. Western blotting analysis confirmed effective knockdown of these cDNAs by the siRNA (Fig. 5*B*). In the absence of TGF-β, none of the siRNA had any effect on Ski stability (Fig. 5*B*), suggesting that none of the E3 ligases that we tested is implicated in the regulation of the basal level of Ski expression. After 40 min of TGF-β stimulation, Ski degradation was completely inhibited by siRNA directed against Arkadia but not by the reduction of Smurf1, Smurf2, or Cdh1 (Fig. 5*B*). Similar results were obtained in A375 melanoma cells (data not shown). These results suggest that although Smurf1 and APC complex are capable of mediating Ski degradation when overexpressed, under physiologic conditions they are not the E3 ligases responsible for Ski degradation. These experiments show that Arkadia mediates TGF-β–induced degradation of Ski in these cancer cell lines.

Smad2 and Smad3 cooperate with Arkadia to mediate TGF-β–induced degradation of Ski. Ski is able to interact with Smad2, Smad3, and Arkadia individually in the absence of TGF-β in transfected cells (33, 43). Because all three molecules are required for Ski degradation in cancer cell lines, we asked whether Smad2 and Smad3 could cooperate with Arkadia to degrade Ski. When overexpressed, wild-type (WT) but not a mutant Arkadia lacking the E3 ligase activity (C937A) was able to induce degradation of Ski (Figs. 5*A* and 6*A*). In contrast, overexpression of either Smad2 or

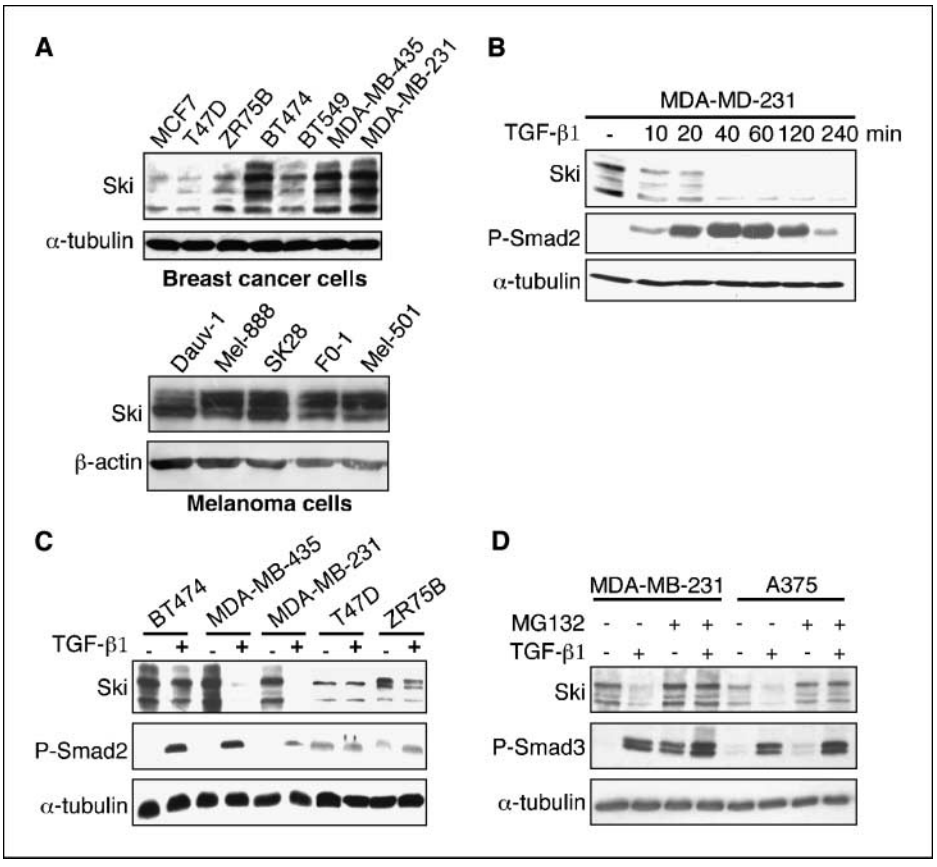

Figure 3. TGF-β induces degradation of Ski through the ubiquitin-dependent proteasome pathway. *A,* expression of Ski in breast cancer cells (*top*) and metastatic melanomas cells (*bottom*). Whole-cell extracts were subjected to Western blotting with anti-Ski antibodies and anti-α-tubulin as a loading control. *B,* expression of Ski in MDA-MB-231 cells in the presence or absence of TGF-β. MDA-MB-231 cells were treated with 100 pmol/L TGF-β1 for the indicated times. Total cellular extracts were analyzed by Western blotting using antibodies against Ski, phosphorylated Smad2 (*P-Smad2*), and α-tubulin. *C,* expression of Ski in various breast cancer cells stimulated with TGF-β1. Breast cancer cell lines were treated or not with 100 pmol/L TGF-β1 for 40 min, and expression of Ski, phosphorylated Smad2, and α-tubulin was analyzed by Western blotting. *D,* TGF-β–induced Ski degradation. Cells were pretreated or not with 50 μmol/L MG132 for 2 h followed by treatment with 100 pmol/L TGF-β1 for 40 min. Whole-cell extracts were analyzed by Western blotting using antibodies against Ski, phosphorylated Smad3 (*P-Smad3*), and α-tubulin.

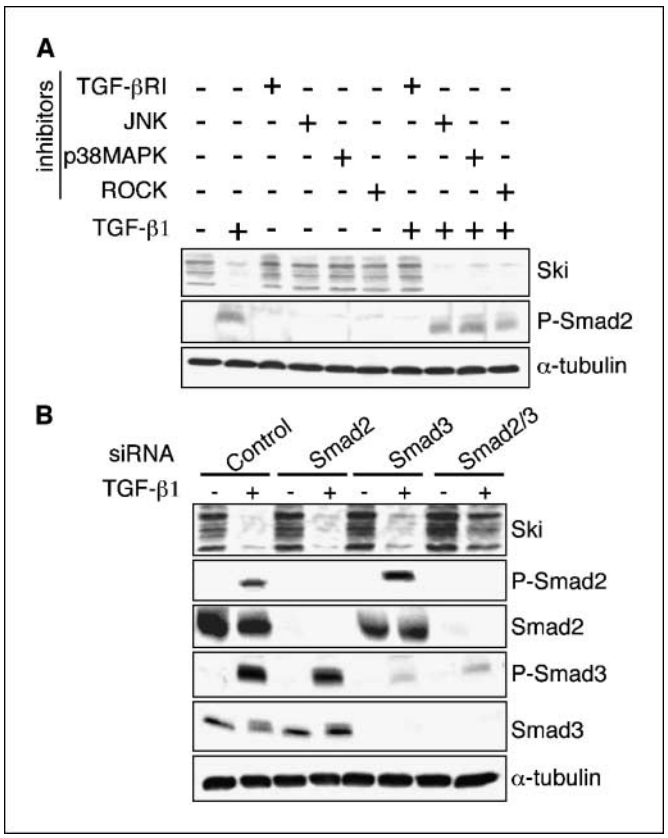

Figure 4. TGF-β induces degradation of Ski through the Smad pathway. *A,* SB-431542 blocked TGF-β–induced degradation of Ski. MDA-MB-231 cells were pretreated for 1 h with either 10 μmol/L SB-431542 (*TGF-βRI*), 10 μmol/L SP600125 (*JNK*), 5 μmol/L SB-20358 (*p38MAPK*), or 10 μmol/L Y-27632 (*ROCK*) followed by treatment with 100 pmol/L TGF-β1 for 40 min. *B,* down-regulation of both Smad2 and Smad3 reduced TGF-β–induced degradation of Ski. MDA-MB-231 cells were transfected with the indicated siRNA. Twenty-four hours after transfection, whole-cell extracts were analyzed by Western blotting using antibodies against Ski, phosphorylated Smad2, Smad2, phosphorylated Smad3, Smad3, and α-tubulin.

Smad3 alone did not reduce Ski level. However, they strongly enhanced the ability of Arkadia to degrade Ski (Fig. 6*A*). This enhancing effect was dependent on the E3 ligase activity of Arkadia as the Smad proteins failed to cooperate with the C937A mutant Arkadia to induce degradation of Ski (Fig. 6*A*).

To further confirm the role of the Smad-Ski interaction in the degradation of Ski, we examined the ability of Arkadia to degrade a mutant Ski that has lost the ability to bind to the Smad proteins (SkimS2/S3,S4W). This mutant could interact with Arkadia as effectively as WT Ski (Fig. 6*B*). However, overexpressed Arkadia, Smad2, and Smad3 failed to reduce the level of this Ski mutant (Fig. 6*C*), confirming that interaction of Smads and Ski is required for its degradation.

Phosphorylation of Smad2 and Smad3 in response to TGF-β stimulation is required for its nuclear translocation and activation. We found that, when overexpressed in 293T cells, Smad2 and Smad3 were phosphorylated at the COOH-terminal serine residues (Fig. 6*D*). To determine whether this phosphorylation is required for Arkadia-mediated degradation of Ski, the Smad3 3SA mutant defective in phosphorylation was used. When overexpressed in 293T cells, Smad3 3SA did not cooperate with Arkadia to degrade Ski (Fig. 6*D*). Thus, binding of phosphorylated Smad2 or Smad3 to Ski is required to induce efficient degradation of Ski by Arkadia.

Discussion

Although both oncogenic and tumor-suppressive functions have been reported for Ski, its exact role in mammalian malignant progression has not been fully defined. In this study, we report that reducing Ski expression in breast and lung cancer cells does not affect tumor growth but enhances tumor metastasis *in vivo*. Consistent with this *in vivo* observation, expression of a panel of metastasis-promoting genes was also up-regulated in tumor cells with reduced Ski expression. Thus, in these cells, Ski seems to have an antitumorigenic activity, implying that high levels of Ski expression in metastatic human cancer cells may serve as a

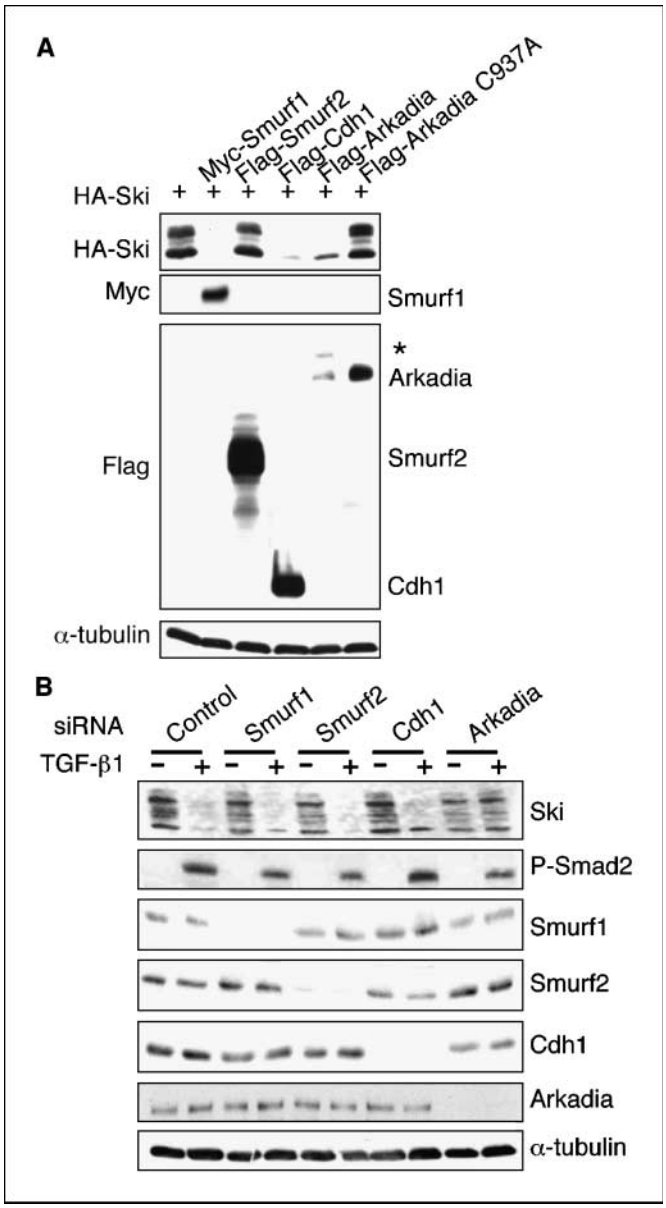

Figure 5. Arkadia mediates TGF-β–induced degradation of Ski. *A,* overexpressed E3 ligases induced degradation of Ski. 293T cells were transfected with Smurf1, Smurf2, Cdh1, and Arkadia as indicated. Twenty-four hours after transfection, Ski expression was analyzed by Western blotting. *, monoubiquitinated form of Arkadia. *B,* knocking down Arkadia inhibited TGF-β–induced degradation of Ski. MDA-MB-231 cells were transfected with the indicated siRNAs. Expression of various proteins was analyzed by Western blotting.

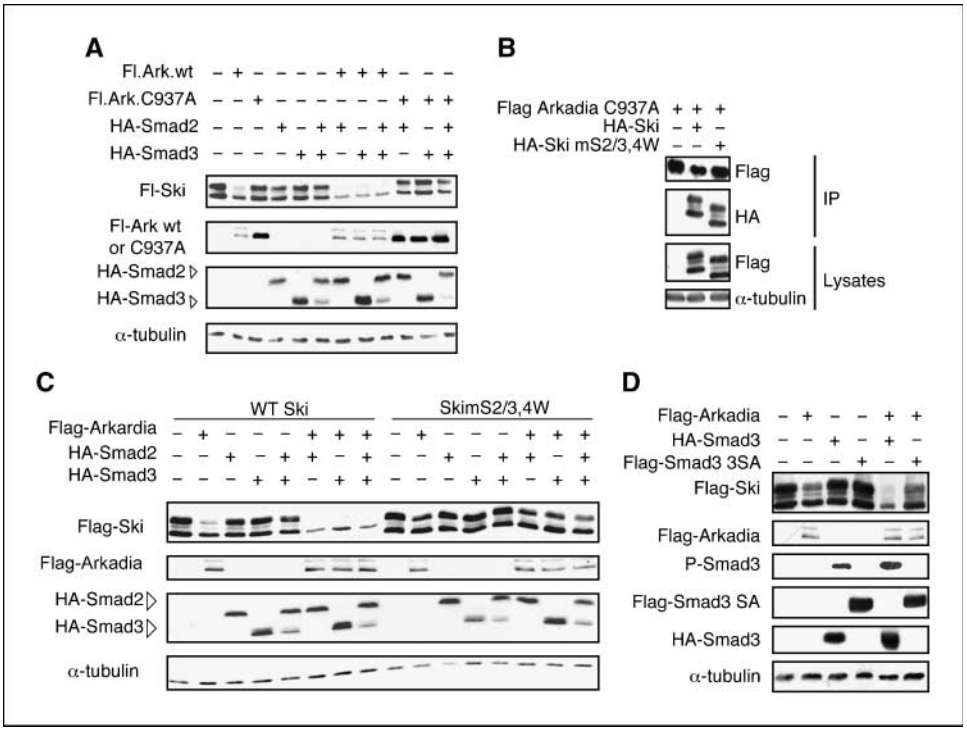

Figure 6. Smad2 and Smad3 cooperate with Arkadia to induce degradation of Ski. *A,* 293T cells were transfected with Flag-Ski, WT or C937A mutant Flag-Arkadia, HA-Smad2, or HA-Smad3 as indicated. Expression of Ski or other proteins was monitored by Western blotting. *B,* mutant Ski deficient in binding to the Smads (SkimS2/S3,S4W) still interacted with Arkadia. 293T cells were transfected with Flag-Arkadia C937A together with WT or mutant HA-Ski as indicated. Ski proteins that associated with Arkadia were isolated by immunoprecipitation (*IP*) with anti-Flag agarose and detected by Western blotting with anti-HA. *C,* SkimS2/S3,S4W was not degraded by Arkadia. 293T cells were transfected with Flag-tagged WT or mutant Ski in the presence or absence of Flag-Arkadia, HA-Smad2, and HA-Smad3 as indicated. Expression of Ski was analyzed by Western blotting. *D,* Smad3 3SA mutant did not cooperate with Arkadia to degrade Ski. 293T cells were transfected with Flag-Ski, Flag-Arkadia, HA-Smad3, or Flag-Smad3 SA as indicated. Degradation of Ski was monitored by Western blotting with anti-Flag.

potential barrier for malignant progression. Understanding the mechanisms by which tumor cells override the effects of Ski and progress toward a metastatic state is of utmost importance. Here, in this report, we provide evidence that TGF-β, a cytokine that is often highly expressed in metastatic tumors, can induce efficient Ski degradation in melanoma and breast cancer cells through the specific recruitment of the E3 ubiquitin ligase Arkadia in a Smad-dependent manner. This ability of TGF-β to induce degradation of Ski could be an additional mechanism contributing to its prometastatic activity. Our work also suggested an important physiologic context under which Ski degradation is regulated by TGF-β.

Contrary to previously reported observations in avian embryo fibroblasts, Ski had little effect on anchorage-independent growth of human cancer cells or on the growth of s.c. tumors *in vivo*. Instead, we found that Ski expression inhibits tumor metastasis. This is different from the closely related SnoN protein, which has a major effect on tumor growth both *in vitro* and *in vivo* (13). In MDA-MB-231 cells that express high levels of both SnoN and Ski, reducing SnoN alone is sufficient to fully block the anchorage-independent growth and tumor formation in nude mice, whereas reducing Ski has no effect. Thus, although Ski and SnoN share redundant functions to induce transformation and terminal differentiation in avian embryo cells, our present study suggests that Ski and SnoN may have different functions in human tumorigenesis.

The inability of Ski to promote tumor growth and its inhibitory activity on tumor metastasis *in vivo* bring forth the question of whether Ski is an oncogene or a tumor suppressor. Presently, the hypothesis that Ski behaves as an oncogene in human cells was supported by studies indicating that the level of Ski expression correlates with the progression of various human cancers, including leukemia, esophageal cancer, pancreatic cancer, and melanoma (4–8). Inversely, the antitumorigenic activity of Ski is also substantiated by the increased susceptibility of heterozygous Ski knockout mice to chemical carcinogens (9), by this study, by its chromosomal localization (10), and by a recent report showing that adenovirus-mediated overexpression of Ski in JygMC(A) mammary carcinoma cells reduced its metastatic potential *in vivo* (44). It is of interest to note that thus far the strongest support for the pro-oncogenic activity of Ski is the ability of Ski to induce transformation of avian embryo fibroblasts (3). It is possible that Ski may be pro-oncogenic in development of tumors of mesenchymal origins, such as fibrosarcoma and liposarcoma, but anti-oncogenic in the development of epithelial-derived tumors. Alternatively, Ski may exert different effects on different aspects or stages of tumorigenesis. Clearly, the function of Ski in mammalian tumorigenesis is more complex than people thought before and needs more investigations.

Ski is an important negative regulator of the Smad proteins. Whether the antimetastatic activity of Ski is related to its ability to inactivate the Smad proteins is yet to be determined. In the microarray analysis, some metastasis- and EMT-related genes that are regulated by Ski are also TGF-β–responsive genes (*SLUG, TNC, HMGA2, SPHK1, ADMATS1*, and *TIMP-3*), whereas others have not been reported to be regulated by TGF-β. Thus, the antimetastatic activity of Ski may be related to both Smad-dependent and Smad-independent pathways.

The inhibitory activity of Ski on tumor metastasis indicates that a mechanism to inactivate Ski must exist in metastatic tumor cells. Indeed, we found that TGF-β can induce efficient degradation of Ski through Arkadia. Although Ski degradation in response to TGF-β has been reported before in transfected cells (34), this regulation did not occur in all cell types, and the physiologic context under which this regulation happens has not been defined. Our results showed that degradation of Ski by TGF-β occurs in metastatic breast cancer and melanoma cells and plays an important role in promoting tumor metastasis. Overexpression of Arkadia has been

shown to induce degradation of Ski in transfected cells (33). However, under similar overexpression conditions, several other E3 ligases (i.e., APC/Cdh1 and Smurf1) also have the same effect. Thus, overexpression experiment alone is not sufficient to determine which E3 ligase is responsible for Ski degradation under physiologic conditions. Using the siRNA approach to reduce the expression of endogenous E3 ligases, we showed that only Arkadia is required for TGF-β–induced degradation of Ski. Furthermore, using a Smad3 mutant that cannot be phosphorylated by TGF-βRI or a Ski mutant that cannot bind the Smad proteins, we found that phosphorylated Smad2 or Smad3 cooperate with Arkadia to degrade Ski. This is consistent with another report by Levy et al. (32) who have shown recently that binding of phosphorylated Smad2 or Smad3 to SnoN is required to induce an efficient degradation of SnoN by Arkadia. Arkadia may also be part of a larger complex that is formed on TGF-β stimulation. Recently, Axin, a central scaffold in the Wnt pathway, has been found to interact with Arkadia to enhance the degradation of Smad7 (45). It is possible that similar scaffold proteins may be required for Arkadia-induced degradation of Ski. Our results show that this regulation can occur in metastatic tumor cells and may play an important role in allowing metastasis to proceed. Thus, our study has revealed a proper biological context for Ski degradation by TGF-β.

Arkadia has recently emerged as a major regulator of the TGF-β signaling through its ability to induce degradation of multiple negative regulators of the pathway, including Ski, SnoN, and Smad7, as well as its ability to enhance transcriptional activation by phosphorylated Smad2 and Smad3 through ubiquitination (32, 33, 46, 47). Although the essential role of Arkadia in normal development has been described (48, 49), its role in tumorigenesis has not yet been defined. By targeting the expression of these negative regulators of TGF-β signaling, Arkadia may enhance the protumorigenic activity of TGF-β in tumor metastasis. Consistent with this, expression of Arkadia was found to increase as renal tubular cells undergo EMT in response to TGF-β (50). Future studies will reveal its role in human carcinogenesis.

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