HHS Public Access

Author manuscript

Pigment Cell Melanoma Res. Author manuscript; available in PMC 2020 May 11.

Published in final edited form as:

Pigment Cell Melanoma Res. 2009 December ; 22(6): 761–772. doi:10.1111/j.1755-148X.2009.00603.x.

SKI knockdown inhibits human melanoma tumor growth *in vivo*

Dahu Chen[1,*], **Qiushi Lin**[1,*], **Neil Box**[2,3], **Dennis Roop**[2,3], **Shunsuke Ishii**[5], **Koichi Matsuzaki**[6], **Tao Fang**[7], **Thomas J. Hornyak**[7], **Jon A Reed**[3], **Ed Stavnezer**[8], **Nikolai A. Timchenko**[1,4], **Estela E. Medrano**[1,2,3,#]

[1]Huffington Center on Aging, Baylor College of Medicine, Houston, TX, USA

[2]Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX, USA

[3]Department of Dermatology, Baylor College of Medicine, Houston, TX, USA

[4]Department of Pathology, Baylor College of Medicine, Houston, TX, USA

[5]Riken Tsukuba Institute, Tsukuba, Ibaraki, Japan

[6]Department of Gastroenterology and Hepatology, Kanasi Medical University, Fumizono-cho, Moriguchi, Osaka, Japan

[7]Dermatology Branch, Center for Cancer Research, NCI, NIH, Bethesda, MD 20892

[8]Case Western Reserve University, Cleveland, OH, USA

Abstract

The SKI protein represses the TGF-β tumor suppressor pathway by associating with the Smad transcription factors. SKI is upregulated in human malignant melanoma tumors in a disease-progression manner and its overexpression promotes proliferation and migration of melanoma cells *in vitro*. The mechanisms by which SKI antagonizes TGF-β signaling *in vivo* have not been fully elucidated. Here we show that human melanoma cells in which endogenous SKI expression was knocked down by RNAi produced minimal orthotopic tumor xenograft nodules that displayed low mitotic rate and prominent apoptosis. These minute tumors exhibited critical signatures of active TGF-β signaling including high levels of nuclear Smad3 and p21$^{Waf\text{\textdiamond}1}$, which are not found in the parental melanomas. To understand how SKI promotes tumor growth we used gain- and loss-of-function approaches and found that simultaneously to blocking the TGF-β-growth inhibitory pathway, SKI promotes the switch of Smad3 from tumor suppression to oncogenesis by favoring phosphorylations of the Smad3 linker region in melanoma cells but not in normal human melanocytes. In this context, SKI is required for preventing TGF-β-mediated down-regulation of the oncogenic protein c-MYC, and for inducing the plasminogen activator inhibitor-1, a mediator

[#]To whom correspondence should be addressed at: Baylor College of Medicine, Houston, TX, 77030 USA Phone: (713) 798-1569 Fax: (713) 798-4161, medrano@bcm.tmc.edu.

[*]These authors contributed equally to this work

Significance

The protein SKI curtails TGF-β growth inhibitory signals by associating with and repressing the activity of C-terminus phosphorylated Smad2/3 complexes. We previously reported that SKI is upregulated in human melanoma tumors in parallel with disease-progression. Here we describe a mechanism by which SKI executes its oncogenic function. We demonstrate that SKI is crucial for Smad3 linker phosphorylations associated with the cancer-promotion trait of the TGF-β signaling pathway in melanoma. SKI prevents down-regulation of c-MYC by TGF-β whereas it simultaneously allows upregulation of the plasminogen-activator inhibitor 1 and repression of p21$^{Waf\text{-}1}$. These results suggest that SKI is a valuable target for melanoma therapies.

of tumor growth and angiogenesis. Together, the results indicate that SKI exploits multiple regulatory levels of the TGF-β pathway and its deficiency restores TGF-β tumor suppressor and apoptotic activities in spite of the likely presence of oncogenic mutations in melanoma tumors.

Keywords

melanoma; SKI; TGF-β; Smad3 linker phosphorylation; c-MYC; PAI-1 Annotation; SKI refers to the human protein; Ski to the mouse protein

Introduction

Cancer cells usually escape growth controls via self-sufficiency in growth-promoting signals (autocrine pathways) and inability to respond to growth-inhibitory signals (Hanahan and Weinberg, 2000). Melanoma tumors are a classical paradigm of such tumor evolution; they produce potent growth factors that enable them to acquire independence from environmental cues needed for proliferation of normal melanocytes (Herlyn and Shih, 1994).

Normal cellular homeostasis is maintained by a variety of growth inhibitory signals that include both soluble factors and matrix-derived inhibitors (Hanahan and Weinberg, 2000). In normal tissues and cells including melanocytes, transforming growth factor β (TGF-β) protects homeostasis by functioning as a tumor suppressor, blocking cell cycle progression via up-regulation of the cyclin-dependent kinase inhibitors p15 and p21Waf-1, and down-regulation of c-MYC (Massague and Gomis, 2006). The Smad proteins are transcription factors that activate gene expression in response to TGF-β. The oncogenic SKI protein blocks TGF-β growth inhibitory activity by associating with the MH2 domains of Smad2 and Smad3 and repressing their transcriptional activity (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999b; Xu et al., 2000). In this context, binding of Ski to Smad3 or Smad4 is necessary and sufficient for repressing TGF-β signaling (Ueki and Hayman, 2003). SKI binds to the N-terminal upper side of the toroidal structure of the Smad3 MH2 oligomer (Mizuide et al., 2003) with a complex stoichiometry that requires exchanging two molecules of the coactivator CBP bound to a pentamer containing receptor-activated Smad2/3/4 by two molecules of SKI (Chen et al., 2007). The SKI/Smad complex, which includes HDAC1, mSin3, N-CoR and other components of repressor complexes, transforms the Smads from activators to repressors of genes associated with the cell cycle (Medrano, 2003). SKI also represses TGF-β via its association with Mel1, which results in the stabilization of Smad3-SKI complexes on TGF-β-regulated promoters (Takahata et al., 2009). In addition, SKI binds to HDAC3 and the protein arginine methyltransferase 5 (PRMT5) to maintain the basal repression state of *Smad7*, a TGF-β-regulated gene (Tabata et al., 2009). SKI, but not snoN, forms a complex with Smad2/4 proteins in melanocytes treated with TGF-β to transcriptionally repress Pax3, a major regulator of the transcription factor MITF (Yang et al., 2008). However, SKI also promotes MITF expression via its association with the adaptor protein FHL2 (Chen et al., 2003). These examples show that SKI inhibits the growth inhibitory pathway of TGF-β through multiple protein partners and mechanisms.

Paradoxically, TGF-β can also act as a tumor promoter by stimulating progression, invasion and metastasis via remodeling the tumor microenvironment and by promoting angiogenesis

(Berking et al., 2001; Miyazono et al., 2003; Pardali and Moustakas, 2007). TGF-β cooperates with RAS to activate JUN NH2-terminal kinase (JNK) resulting in the phosphorylation of the linker regions of Smad2 and Smad3. Such phosphorylations induce the reversible switch from tumor suppression to oncogenesis by upregulating c-MYC, PAI-1 and matrix metalloproteinases involved in tumor invasion (Sekimoto et al., 2007). In this regard, most human melanoma tumors display activating mutations in the RAS or BRAF signaling pathways (Haluska et al., 2006). Constitutively active ERK increases c-Jun transcription resulting in a feed-forward activation of the JNK-JUN pathway that leads to elevation of cyclin D1 and other genes frequently overexpressed in human melanoma (Lopez-Bergami et al., 2007).

Human melanoma tumors and cells secrete to the microenvironment high levels of TGF-β (Berking et al., 2001); however, they are poorly or not responsive to TGF-β-mediated growth inhibition (Medrano, 2003; Rodeck et al., 1994). The oncogenic protein SKI is upregulated in human melanoma in parallel with disease-progression, promoting down-regulation of $p21^{Waf-1}$ (Reed et al., 2001) and up-regulation of MITF and Nr-CAM, two proteins associated with melanoma cell survival, growth, motility, and transformation (Chen et al., 2003). In primary melanomas, nuclear SKI is associated with Breslow thickness and ulceration, a condition displayed by aggressive tumors (Boone et al., 2009). In advanced melanoma tumors, SKI can localize to both nucleus and cytoplasm or cytoplasm only (Reed et al., 2001). When localized to the cytoplasm, SKI can also restrict TGF-β growth inhibitory activities by binding and sequestering TGF-β-activated Smads and preventing their nuclear localization (Kokura et al., 2003; Reed et al., 2001). Thus, resistance of melanoma cells to growth inhibition by their own secreted TGF-β is mediated by the presence of high levels of the nuclear/cytoplasmic SKI, and not by a defective TGF-β receptor signaling.

Here we have asked whether these actions of SKI contribute to melanoma tumor formation. We demonstrate that knocking down SKI by double strand RNA expression restores anchorage dependent growth and dramatically impairs melanoma xenograft growth *in vivo*; apparently by reactivation of TGF-β growth inhibitory and apoptotic pathways. Additional data supporting these findings indicate that SKI executes multiple roles as sensor and modifier of TGF-β signaling by preventing full nuclear localization of Smad2/3, tethering C-terminus phosphorylated Smads to transcriptional repressor complexes, and promoting linker region phosphorylations of Smad3 that are associated with the shift of its activity from tumor suppression to oncogenesis (Matsuzaki, 2006).

Results

Down-regulation of SKI by ds-RNA Restores Anchorage-dependent Growth of Human Melanoma Cells

The levels of SKI expression correlates with the progression of human melanoma from primary invasive to metastatic tumors (Reed et al., 2001) and its overexpression in human melanoma cells and mouse *Ski−/−*melanocytes results in increased proliferation, clonogenicity and motility (Chen et al., 2003). Confirming these results, SKI is highly expressed in several human melanoma cell lines derived from primary and metastatic tumors

whereas it is virtually absent in normal human melanocytes (Supplemental Fig. 1A). To define the role of SKI in human malignant melanoma growth and survival *in vivo*, we used pDECAP vectors for SKI knockdown. This RNAi technology was previously shown to be highly efficient in down-regulating mouse Ski *in vivo* (Shinagawa et al., 2001). This strategy was also highly efficient in down-regulating SKI in three human melanoma cell lines, UCD-Mel-N, A375 (Figs.1A and 1B) and IIB-Mel-N (data not shown). Down-regulation of SKI did not significantly alter their proliferation rates when cultured under regular conditions in plastic dishes (data not shown). Yet, major differences between control melanoma and RNAi-SKI cell lines emerged when the cells were cultured in the absence of a substratum. Down-regulation of SKI significantly reduced colony formation in agar (Figs. 1A and 1B). The failure of these cells to form large number of colonies in soft agar was due to down-regulation of SKI and not to off target of double strand RNA effects because re-expression of SKI in UCD (RNAi-SKI) cells restored the anchorage-independent phenotype to that of melanoma cells expressing endogenous SKI (Fig. 1A, SKI-EV cells). These results are consistent with previous data showing that SKI increases both motility and clonogenicity of human melanoma cells (Chen et al., 2003).

Endogenous TGF-β signaling appears to be responsible for the constitutive levels of activated, C-terminus phosphorylated Smad proteins observed in a variety of human melanoma cell lines (Rodeck et al., 1999). We and others have previously demonstrated that binding of SKI to receptor-activated Smad2/3 and Smad4 results in the formation of protein complexes that blunt TGF-β-mediated growth inhibition (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999a; Xu et al., 2000). When localized to the cytoplasm, SKI suppresses TGF-β signaling by sequestering receptor-activated Smads and preventing their nuclear translocation (Kokura et al., 2003; Reed et al., 2001). Therefore, we assessed the effect of SKI-knockdown on the TGF-β response by determining the intracellular localization of the Smad2/3 proteins in UCD-EV and UCD (RNAi-SKI) cells. In control UCD(EV) cells cultured in the absence of exogenous TGF-β Smad2/3 were mostly localized to the cytoplasm (Fig. 1C, upper left panel). In contrast, Smad2/3 localized to both cytoplasm and nucleus in SKI knockdown cells (Fig. 1C lower left).

Recently, it has been shown that high concentrations (0.1 μM) of TGF-β down regulate SKI, inhibiting its ability to induce tumor metastasis in two cell lines derived from breast and lung cancer (Le Scolan E. et al., 2008). Similar supra-physiological levels of TGF-β-can also downregulate SKI in A375 melanoma cells (Supplemental Fig. 1B). However, SKI is minimally degraded by lower concentrations (8–80pM) of TGF-β in melanoma cells (Supplemental Fig. 1C and 1D). These results are in agreement with previous reports showing constitutive expression of SKI in primary and metastatic human melanoma tumors (Boone et al., 2009; Reed et al., 2001), and resistance of melanoma cell lines to the growth inhibitory effects of TGF-β (Berking et al., 2001) regardless of TGF-β produced by the microenvironment and/or by melanoma cells. Of most importance, low TGF-β concentrations were physiologically relevant, as they induced significant Smad2 and Smad3 phosphorylations (Supplemental Fig. 1C and 1D). Consistent with this observations, exposure to 0.6 ng/ml (24pM) TGF-β for two hrs resulted in partial Smad2/3 nuclear localization in UCD-EV cells, whereas the same treatment induced full nuclear localization of Smad2/3 in UCD(RNAi-SKI) cells (Fig. 1C (compare upper right with lower right

panel)). Together these results indicate that SKI is responsible for altering Smad2/3 localization in melanoma cells.

It was previously shown, that 0.5ng/ml TGF-β minimally inhibited the growth of A375 and other melanoma cell lines (Krasagakis et al., 1999). We also observed that A375 and UCD-Mel-N minimal growth inhibition when cultured in the presence of 0.6 ng/ml TGF-β (Supplemental Fig. 1E) regardless of the presence of high pSmad2 and pSmad3 levels. Using UCD(RNAi-SKI) cells as a reference, we also confirmed previous data showing that even when overexpressed (UCD-SKI+) SKI is not downregulated by 0.6ng/ml TGF-β (Supplemental Fig. 2A), and does not substantially change TGF-β-induced, pSmad2 or pSmad3 C-terminus phosphorylations (Supplemental Fig. 2A). However, its presence is required for the formation of Smad2/3 repressive complexes containing mSin3, HDAC1 and RB (Supplemental Fig. 2, compare B–C with D–E).

SKI-depleted melanoma cells form minute xenografts with reactivation of TGF-β signaling

Because the *in vitro* analysis did not take into account the interaction of the melanoma cells with the tumor microenvironment, we assessed the effect of *SKI* knockdown on tumor formation *in vivo.* We injected 1×10^6 UCD-EV cells and 3×10^6 UCD(RNAi-SKI) cells orthotopically into nude mice and monitored tumor growth of subcutaneous tumors by daily examination and bi-weekly measurements with calipers. It was previously demonstrated that the parental, wild-type UCD-Mel-N cells form large xenograft tumors in immuno-compromised mice (Garcia et al., 2004). Comparisons between time-matched tumors showed that whereas UCD-EV cells yielded tumors with 100% efficiency, reaching ~400 mm3 in mean volume after 27 days, UCD(RNAi-SKI) produced minute, apoptotic tumors, even when using three times more cells than the controls (Fig. 2A). Pathological assessment of the tumors showed that down-regulation of SKI resulted in remarkable differences in morphology and mitotic rates (Fig. 2A and Supplemental Table 1). Control tumor cells (EV) universally had a large epithelioid or spindle-shaped morphology (Fig. 2B, a and c). These cells produced large tumor nodules with high mitotic rates and small central zones of cellular necrosis (Table 1, mean 18/mm2). Conversely, (RNAi-SKI) cells yielded small tumor nodules containing small epithelioid cells, prominent central zones of cellular apoptosis/necrosis (Fig. 2B, b and d), and a significantly lower mitotic rate (Table 1, mean 2.33 / mm2) compared to (EV) tumors. Comparisons between size-matched (tumor volume $<20mm^3$) early control tumors (n=6 animals at 3 days after injection) with SKI knockdown tumors (n=6 animals at 10 days after injection) showed no statistical differences in apoptosis and number of mitosis (data not shown). This suggests that whereas (EV) tumors begin to exponentially grow shortly after cell implantation, RNAi-SKI tumors develop at a similar rate after an initial lag but then fail to thrive and become apoptotic. Immunohistochemistry confirmed that nuclear SKI was expressed in (EV) tumor cells whereas only a few scattered (RNAi-SKI) cells showed SKI immunoreactivity (Fig. 2C, a and b). Since TGF-β increased the nuclear localization of Smad2/3 in RNAi-SKI melanoma cells in culture (Fig. 1D), we investigated whether such activity was reactivated in the small, apoptotic tumors. We found that Smad3 was prominently detected in the nucleus and cytoplasm of the minute RNAi-SKI tumors (Fig. 2C–d). In contrast, Smad3 was mostly detected in the rim of EV tumor cells

(Te) and host stromal (S) cells (Fig. 2Cc), whereas it was below detection in the tumor mass (T).

In cells responsive to TGF-β signaling, activated Smad3 binds the FoxO transcription factors to activate transcription of $p21^{Waf-1}$ (Seoane et al., 2004). Consistent with these data, nuclear-localized $p21^{Waf-1}$ was prominently detected in the Smad3-expressing (RNAi-SKI) tumor cells surrounding the central zone of necrosis (Fig. 2Cf) star). In contrast, only a few scattered cells expressed $p21^{Waf-1}$ in the large, (EV) tumor cells. Together, these experiments provide evidence for the role of SKI in preventing Smad-mediated growth inhibitory and/or apoptotic pathways in melanoma, a tumor conspicuously resistant to TGF-β pathways associated with tumor suppression (Rodeck et al., 1994; Rodeck et al., 1999). Our results support and extend previous data using pancreatic cancer cell line xenografts injected three times/week for 2weeks with RNAi-SKI. Tumor necrosis was evident at the sites of injections but neither Smad3 nor $p21^{Waf-1}$ were assessed in the xenografts (Heider et al., 2007).

Endogenous SKI enhances pro-oncogenic phosphorylations in the linker region of Smad3

Dephosphorylation of the linker region of Smad2 and Smad3 by small C-terminus phosphatases enhances TGF-β growth inhibitory pathways (Sapkota et al., 2006; Wrighton et al., 2006). In contrast, RAS/JNK-mediated linker phosphorylations of Smad2 and Smad3 results in activation of TGF-β pathways associated with matrix degradation and invasion including up-regulation of c-MYC, the plasminogen activator inhibitor-1 (PAI-1) and the matrix metalloproteinases MMP-1, MMP-2 and MMP-9 (Sekimoto et al., 2007). Having established the role of SKI in Smad3 nuclear localization and tumor formation, we wished to investigate whether SKI was acting solely as a Smad repressor, or whether, in addition, it might also function to activate the pro-oncogenic activity of Smad3 via linker phosphorylation. To test this role, we assessed Smad phosphorylations in UCD-Mel-N and A375 wild-type and derivatives expressing an empty vector or RNAi-SKI, and normal human neonatal and adult melanocytes. The aforementioned tumor cell lines were chosen because they likely represent two different melanoma subgroups; UCD-Mel-N has an activating mutation, Q61R, at *NRAS* codon 61 (Supplemental Fig. 3), whereas A375 displays the $BRAF^{V600E}$ activating mutation (Satyamoorthy et al., 2003), and constitutively active ERK and JNK kinases (Lopez-Bergami et al., 2007). TGF-β induced robust, SKI-independent, C-terminus Smad2 and Smad3 phosphorylations in both melanoma cells and melanocytes (Figs. 3Ab and 3Bb). Yet, significant differences were observed in Smad3 linker phosphorylations associated with TGF-β-pro-oncogenic pathways. $Smad3L^{208–213}$ phosphorylations were minimally detected in RNAI-SKI melanoma cells (Fig. 3Af) or in normal human melanocytes of either neonatal or adult origin (Fig. 3Bf). $Smad3L^{208–313}$ phosphorylations are SKI-dependent since its re-expression in UCD-(RNAi-SKI) cells restored the robust linker phosphorylations (Fig. 3Cc, compare lane 4 with lane 6) observed in EV controls (Fig. 3Cc, lane 2) after TGF-β treatment. Basal phosphorylation of $Smad3L^{208–213}$ (Figs. 3Af, lanes 1 and 3, and 3Bf, lane5) may result from the convergence of several pathways including autocrine stimulation by melanoma-secreted TGF-β and activation of JNK and ERK kinases via RAS or BRAF mutations (discussed in more detail in the next section).

SKI dual actions in the TGF-β pathway: it prevents downregulation of c-MYC and stimulates upregulation of PAI-1

Melanoma tumors and cell lines display constitutively activated ERK- and a rewired JNK-signaling pathways that result in increased expression of c-Jun, cyclin D1 and RACK1 (Lopez-Bergami et al., 2007; Reed et al., 2001). TGF-β induces JNK activation by both Smad-dependent and -independent pathways; rapid, Smad-independent JNK activation by TGF-β is followed by Smad-dependent JNK activity that can last several hours (Engel et al., 1999). To test whether downregulation of SKI affects the TGF-β/JNK/ERK pathway, we compared pJNK and pERK levels between normal melanocytes, UCD-Mel-N and A375 melanoma cells. Basal levels of pJNK1 were higher in UCD-Mel-N compared with A375 cells (Fig. 4Ab). Downregulation of SKI, or treatment with TGF-β, did not significantly change pJNK1 in UCD-Mel-N cells, and minimally increased it in A375 cells. In contrast, constitutively active JNK2 (pJNK2) was observed in UCD-Mel-N but not after SKI downregulation, or in A375 cells. In addition, pERK levels were similar between normal melanocytes, and the two melanoma cells lines. Together, the results suggest that SKI doesn't regulate JNK1 or ERK.

TGF-β is a potent negative regulator of c-MYC expression (Reviewed in (Siegel and Massague, 2008)). c-MYC is induced by hyperactive RAS and is required for suppression of senescence in melanoma cells (Zhuang et al., 2008). Consistent with previous data (Zhuang et al., 2008), c-MYC levels were below detection in normal melanocytes (Fig. 4Ah). In contrast, c-MYC was highly expressed in UCD-Mel-N and A375 cells, (Fig. 4Ah). Significantly, we found that SKI is required for preventing downregulation of c-MYC by TGF-β in both UCD and A375 melanoma cells (Fig. 4Ah, arrowheads). These results are biologically relevant for the cell's fate, as downregulation of c-MYC by RNAi results in cell cycle arrest in the G_0/G_1 phase of the cell cycle of human melanomas cells (Wang et al., 2008).

To analyze in more detail the role of pSmad3L phosphorylations in melanoma cells, we performed a kinetic analysis of SmadC/L phosphorylations after TGF-β treatment in A375 cells. In agreement with data shown in Supplemental figure 2, Smad2C and Smad 3C phosphorylations showed little or no changes in A375 SKI-KD compared to EV cells (Fig. 4Bc and 4Bf). Consistent with experiments shown in Fig 3, SKI was required for robust Smad3L phosphorylations after TGF-β-treatment (Fig. 4Bg, underlined). Increased levels of PAI-1 are associated with a poor prognosis in human cancers (McMahon et al., 2001). An hyperactive RAS pathway can stimulate Smad3L phosphorylations, which fosters tumor invasion by up-regulating PAI-1 (Sekimoto et al., 2007). In agreement with with this data, SKI and pSmad3L correlate with induction of PAI-1 in A375 EV melanoma cells (Fig. 4Bj, lanes 10 to 15). We also noted a late, SKI-independent, upregulation of PAI-1 (compare Fig. 4Bj lanes 8 with 16). Such up-regulation may result via autocrine activity mediated by TGF-β and/or other melanoma-derived growth factors accumulated in the culture medium. SKI prevented basal and TGF-β-regulated $p21^{Waf-1}$ expression (Fig. 4Bk and l). Together, these results indicate that SKI levels correlate with Smad3L phosphorylations, resistance to c-MYC downregulation by TGF-β, repression of $p21^{Waf-1}$ and up-regulation of PAI-1; three essential components of many cancer progression pathways.

Discussion

SKI is required for the growth of orthotopic human melanoma xenograf tumors

TGF-β has dual functions as tumor suppressor and tumor promoter (Dumont and Arteaga, 2003). Whereas some tumors escape the cytostatic activity of TGF-β by acquiring mutations in its receptors, or in members of its signaling pathway including the Smad proteins, other tumors such as gliomas depend on TGF-β/Smad activity for proliferation (Bruna et al., 2007). Human melanoma tumors display wild-type TGF-β receptors (Schmid et al., 1995) and a responsive Smad signaling pathway (Rodeck et al., 1999). Human melanocytes are potently growth inhibited by TGFβ whereas most melanoma cells progressively lose their responsiveness to TGF-β in the advanced vertical growth phase in the skin, and are growth stimulated by this cytokine when they metastasize to other organs (Krasagakis et al., 1993; Krasagakis et al., 1995; Krasagakis et al., 1999; MacDougall et al., 1993; Reed et al., 1994; Rodeck et al., 1994; Yang et al., 2008). Consistent with this data, melanoma cells express a number of TGF-β-regulated genes involved in invasion and metastasis including adhesion receptors and PAI-1 (Reviewed in (Javelaud et al., 2008)). Based on the dichotomy of TGF-β-activity (growth inhibitor and cancer promoter), and downregulation of SKI by high concentrations of TGF-β in some melanoma cell lines, it was proposed that SKI may not repress TGF-β-signaling in melanoma cells (Javelaud et al., 2008; Le Scolan E. et al., 2008). Our data show that a) SKI is critical for melanoma tumor growth; b) SKI is not downregulated in melanoma xenografts, although Smad3 immunoreactivity in the rim of the tumors suggests an active TGF-β signaling pathway (Fig. 2). This result is consistent with high levels of SKI in metastatic melanoma (Reed et al., 2001), regardless of TGF-β produced by melanoma cells and their microenvironment (Berking et al., 2001); c) SKI amplifies the pro-carcinogenic role of TGF-β by promoting Smad3L phosphorylations associated with tumor progression.

The functional role of SKI in TGF-β signaling: repressing tumor suppression and promoting proliferation and invasion

SKI responds to TGF-β by forming Smad repressive complexes containing SKI, HDAC1, mSin3 and RB (Supplemental Fig.2). Down-regulation of SKI results in loss of anchorage-independent growth in melanoma cell lines and in inhibition of melanoma tumor growth in immunocompromized animals (Fig. 2). These data suggest that failure to activate Smad-regulated growth inhibitory genes including $p21^{Waf\text{-}1}$ in response to TGF-β produced by the tumor microenvironment, or by melanoma autocrine pathways, allows these tumors to benefit from the matrix remodeling, angiogenesis and tumor promotion activities induced by this cytokine (Fig. 5).

SKI, like TGF-β, has dual functions as a tumor suppressor and oncogene (Pardali and Moustakas, 2007). In association with Six1 and Eya3, SKI promotes muscle differentiation by transcriptionally activating myogenin (Zhang and Stavnezer, 2008) whereas its downregulation reduced metastasis of two cell lines (MDA-MB-231 and A549) derived from breast and lung cancers (Le Scolan E. et al., 2008). In addition, $Ski^{-/+}$ animals display increased susceptibility to tumorigenesis (Shinagawa et al., 2001). In contrast to these results, SKI is up-regulated in a disease-progression manner in melanoma (Boone et al.,

2009; Reed et al., 2001), esophageal cancer (Fukuchi et al., 2004), pancreatic cancer (Heider et al., 2007), hepatocellular cancer (Longerich et al., 2004), and acute myeloid leukemia (Teichler et al., 2008). These results suggest that SKI's activities are cell-, tissue-, and tumor-dependent. Supporting this hypothesis, recent data showed that SKI levels and cellular localization regulate the cell-type specific effects of TGF-β. For example, TGF-β downregulates SKI in epithelial cells but not in Schwann cells, inducing in this case RB phosphorylation and loss of TGF-β mediated growth arrest (Jacob et al., 2008), which is virtually identical to the response of melanoma cells expressing high levels of SKI (this communication).

A DNA microarray analysis demonstrated that 86 melanoma cell lines can be grouped in three different cohorts (A–C). Differential gene expression suggests that transition from a weakly (A) to strongly (C) metastatic phenotype correlates with expression of TGF-β-regulated genes that are associated with invasion and metastasis including N-cadherin, collagen type 5, fibroblast growth factor, interleukin 6, thrombospondin, tumor necrosis factor receptor 11b, tropomyosin 1 and 2 and PAI-1 and Wnt signaling inhibitors (Hoek et al., 2006). Our results are in agreement with that data, and also support and extend previous studies describing the complex roles of Smad3 in apoptosis and carcinogenesis. For example, Smad3 null mice are resistant to skin carcinogenesis protocols (Li et al., 2004). In colorectal cancers, phosphorylations at amino acids 208 and 213 in the Smad3 linker region are associated with advanced disease and metastasis (Yamagata et al., 2005). In contrast, weakly metastatic tumors are enriched in β-catenin regulated genes including *SOX-10* and *MITF* and do not express genes associated with the TGF-β oncogenic pathway (Hoek et al., 2006). Thus, by comparing side-by-side Smad2 and Smad3 phosphorylations at the C-terminus and linker regions between melanomas with and without SKI with normal melanocytes, we identified Smad3L as a major SKI target. In this manner SKI reads, executes and diversifies pro-oncogenic activities by preventing TGF-β-mediated downregulation of c-MYC and promoting upregulation of PAI-1. c-MYC plays many roles in both gene activation and gene repression. It expression is positively associated with cell growth (mass) and proliferation, whereas it negatively regulates genes involved in cell cycle inhibition including cyclin-dependent kinases, cell adhesion molecules, anti-apoptotic and differentiation genes (Eilers and Eisenman, 2008). In turn PAI-1, which is not induced by c-MYC (Watson et al., 2002), is a predictor of poor clinical outcome in breast, colon and neuroblastoma (Andreasen, 2007). PAI-1 protects cells from FasL-mediated apoptosis (Bajou et al., 2008), a feature highly relevant for melanoma because this tumor is notoriously resistant to conventional therapies.

Mutations of the Smad3 linker phosphorylation sites activate the growth inhibitory function of this protein in response to TGF-β (Wang et al., 2009) Notably, Smad2 and Smad3 phosphorylated at both the C-terminus and linker regions transduce pro-oncogenic TGF-β signals in late stage human colon cancer (Matsuzaki et al., 2009). It remains to be determined how SKI levels regulate Smad3L linker phosphorylations in melanoma. Binding of SKI to C-terminus phosphorylated Smad3 may prime this molecule for phosphorylations via the TGF-β, RAS/BRAF and JNK pathways in the linker region. It is also possible that SKI itself may be a target of phosphorylations by these pathways, which could lead to increased affinity for Smad3. Alternatively, binding of SKI with Smad3 may impair rapid

dephosphorylation of amino acids 208 and 213 by specific phosphatases (Lin et al., 2006; Sapkota et al., 2006). The linker region of Smad3 protrudes from the N-terminal upper side of the MH2 oligomer toroid (Mizuide et al., 2003). Although SKI binds to two short segments of Smad3 (amino acids QPSMT and SE), a yeast two-hybrid screen using SKI as a bait yielded Smad3 clones containing a segment of the linker region (amino acids 211–220), in addition to the MH2 domain (Xu et al., 2000). This suggests that SKI could partially "shadow" the phosphorylated amino acids 208 and 213 from the action of phosphatases by a steric hindrance effect.

In summary, results from testing the biological functions of SKI *in vivo* and from biochemical approaches *in vitro* support a pro-oncogenic role of SKI in melanoma. The data suggest that SKI is a good candidate for novel melanoma therapies.

Material and Methods

Cell culture

Primary cultures of normal human neonatal and adult melanocytes, and melanoma cell lines were cultured as previously described (Bandyopadhyay et al., 2007). The human melanoma cell lines SB2, A375, Mel888 and DM4 were kindly provided by Menashe Bar-Eli. The human melanoma cell lines UCD-Mel-N and a derivative overexpressing the human *SKI* gene were described previously (Garcia et al., 2004; Xu et al., 2000)

Plasmids

The pDECAP-ski plasmid which expresses SKI double strand (ds) RNA was constructed by inserting PCR amplification products from the human SKI cDNA coding region (amino acid residues 1–261) into pDECAP vector as an inverted repeat with a 12bp spacer (GGTGCGCATATG) (Shinagawa et al., 2003). The SV40 promoter plus hygromycin cDNA and poly A sequences from pcDNA3.1 vectors (Invitrogen) were inserted into the pDECAP-SKI plasmid. Stable colonies were selected for hygromycin resistance.

Immunofluorescence and Immunohistochemistry

pDECAP (EV) and pDECAP (SKI) cells were seeded in coverslips in 6-well plates and cultured in Dulbecco/F12 containing 10% fetal bovine serum. The next day, the medium was replaced with serum free medium. After 48h the cells were treated with 2ng/ml TGF-β1 for 0 or 1h, fixed with 10% formaldehyde , washed with PBS and permeabilized in 1% Triton X-100 in PBS for 20 min. After incubation with 5% bovine serum albumin in PBS containing 0.05% Tween 20 (PBST), the cells were exposed to an anti-Smad2/3 antibody (Ab 3102, Cell Signaling, Danvers, MA) followed by incubation with a goat anti-rabbit Alexa Fluor 495 (A11012, Invitrogen, Carlsbad, CA) secondary Ab. After a final wash, the slides were mounted with anti-fade mounting media containing DAPI (H-1200, Vector Laboratories, Inc., Burlingame, CA) and observed with a fluorescence microscope. Heat-induced epitope retrieval was performed by submerging the sections in Nuclear De-Cloaker solution, pH 9.5 (BioCare Medical, Concord, CA) for 20 min. in a steam bath followed by cooling at room temperature (RT). The primary commercial antibodies (rabbit polyclonal anti-Smad 3, 1μg/ml, Zymed Laboratories, Carlsbad, CA; Mouse anti- p21waf-1, clone

DCS-60.2, 4 μg/ml, Lab Vision, Fremont, CA) were applied to the sections for 16 hr at 4°C, localized by the detection system (Mach 4 polymer, BioCare Medical, 30 min., at room temperature (RT)), Incubation in chromogen solution (Nova Red, Vector Laboratories, Burlingame, CA, was performed for 5 min., RT). Negative controls were performed in the absence of primary antibodies with anti-rabbit or anti-mouse IgG. SDS-PAGE-Western blot analysis was performed as previously described (Reed et al., 2001).

Western blotting and linker-specific anti-phospho Smad2 and anti-phospho Smad3 Abs

Western blotting: Cells were lysed in a 10 mM Tris-Cl (pH 8.0) buffer containing 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS 140 mM NaCl, 1mM PMSF plus a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail set II (Calbiochem). The polyclonal anti-pSmad2L (S250–255), a mixture of anti-Smad3L(S208) and anti-Smad3L(213) antibodies (Sekimoto et al., 2007), and a monoclonal G8 anti-Ski Ab (Colmenares et al., 1991) were used as described previously. The following commercial Abs were used following manufacturer's instructions: polyclonal Abs Smad2 (Invitrogen, 51–1300); Smad3 (Invitrogen, 51–1500), polyclonal SKI Ab (Santa Cruz, sc-9140), HDAC1 (Santa Cruz, sc-7872), mSin3 (Santa Cruz, sc-994); pSmad2C (Ser 465–467) (Abcam, Ab5451), pSmad3C (Ser423–425, Cell Signaling, Ab 3108); polyclonal anti-p21(Abcam, Ab 7960), polyclonal anti-c-MYC (A-14) (Santa Cruz, sc-789), monoclonal anti- PAI-1 (BD Bioscience Ab 612024). ERK: Rabbit, Santa Cruz,(sc-93), monoclonal pERK (Santa Cruz, sc-7383), polyclonal c-MYC (Santa Cruz, sc-789) polyclonal JNK (Cell Signaling, 9252), polyclonal phospho-SAPK/JNK (Thr183/Tyr185- Cell Signaling, 9251), monoclonal PAI-1 (BD Transduction Laboratory, 612024).

Colony Formation in Agar Plates

4×104 pDECAP(EV) and 4×104 pDECAP(RNAi-SKI) cells were seeded in 60 mm dishes containing 0.3% top and 0.7% bottom low melt agarose. Every 3 days, 0.1 ml of fresh medium was added to the dishes to prevent dehydration. After 15 days, the colonies were stained overnight with a solution containing 1mg/ml p-iodotetrazolium violet.

Animals

Male athymic BALB/c nude mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 6 weeks of age. Animals were maintained in state of the art facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

Xenograft Tumor Growth

Cells in exponential growing were harvested, washed with Dulbecco/F12 containing 10% fetal bovine serum and resuspended in Ca2+/Mg2+-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion and only single-cell suspensions of >90% viability were used in the xenograft studies. S.c. tumors were produced by injection of 1×10^6 UCD(EV) cells and 3×10^6 UCD(RNAi-SKI) cells in 0.1 ml of

HBSS over the scapular region. After euthanizing the animals, tumors were collected for their histological assessment and antibody staining.

Statistical Analysis

The in vivo data were analyzed for significance by using SSPS® software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the National Institutes of Health grants 2RO1CA084282 (E.E.M) and RO1CA10070 (N.A.T), and in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research (T.F and T.J.H).

Reference List

Akiyoshi S, Inoue H, Hanai J, Kusanagi K, Nemoto N, Miyazono K, Kawabata M. c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J. Biol. Chem. 1999; 274:35269–35277. [PubMed: 10575014]

Andreasen PA. PAI-1 - a potential therapeutic target in cancer. Curr. Drug Targets. 2007; 8:1030–1041. [PubMed: 17896954]

Bajou K, Peng H, Laug WE, Maillard C, Noel A, Foidart JM, Martial JA, DeClerck YA. Plasminogen activator inhibitor-1 protects endothelial cells from FasL-mediated apoptosis. Cancer Cell. 2008; 14:324–334. [PubMed: 18835034]

Berking C, Takemoto R, Schaider H, Showe L, Satyamoorthy K, Robbins P, Herlyn M. Transforming Growth Factor-beta1 Increases Survival of Human Melanoma through Stroma Remodeling. Cancer Res. 2001; 61:8306–8316. [PubMed: 11719464]

Boone B, Haspeslagh M, Brochez L. Clinical significance of the expression of c-Ski and SnoN, possible mediators in TGF-beta resistance, in primary cutaneous melanoma. J. Dermatol. Sci. 2009; 53:26–33. [PubMed: 18782659]

Bruna A, Darken RS, Rojo F, Ocana A, Penuelas S, Arias A, Paris R, Tortosa A, Mora J, Baselga J, Seoane J. High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. Cancer Cell. 2007; 11:147–160. [PubMed: 17292826]

Chen D, Xu W, Bales E, Colmenares C, Conacci-Sorrell M, Ishii S, Stavnezer E, Campisi J, Fisher DE, Ben Ze'ev A, Medrano EE. SKI activates Wnt/beta-catenin signaling in human melanoma. Cancer Res. 2003; 63:6626–6634. [PubMed: 14583455]

Chen W, Lam SS, Srinath H, Schiffer CA, Royer WE Jr, Lin K. Competition between Ski and CREB-binding protein for binding to Smad proteins in transforming growth factor-beta signaling. J. Biol. Chem. 2007; 282:11365–11376. [PubMed: 17283070]

Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. Cancer Cell. 2003; 3:531–536. [PubMed: 12842082]

Eilers M, Eisenman RN. Myc's broad reach. Genes Dev. 2008; 22:2755–2766. [PubMed: 18923074]

Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J. Biol. Chem. 1999; 274:37413–37420. [PubMed: 10601313]

Fukuchi M, Nakajima M, Fukai Y, Miyazaki T, Masuda N, Sohda M, Manda R, Tsukada K, Kato H, Kuwano H. Increased expression of c-Ski as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma. Int. J. Cancer. 2004; 108:818–824. [PubMed: 14712482]

Garcia M, Fernandez-Garcia NI, Rivas V, Carretero M, Escamez MJ, Gonzalez-Martin A, Medrano EE, Volpert O, Jorcano JL, Jimenez B, Larcher F, Del RM. Inhibition of xenografted human melanoma growth and prevention of metastasis development by dual antiangiogenic/antitumor activities of pigment epithelium-derived factor. Cancer Res. 2004; 64:5632–5642. [PubMed: 15313901]

Groppe J, Hinck CS, Samavarchi-Tehrani P, Zubieta C, Schuermann JP, Taylor AB, Schwarz PM, Wrana JL, Hinck AP. Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol. Cell. 2008; 29:157–168. [PubMed: 18243111]

Haluska FG, Tsao H, Wu H, Haluska FS, Lazar A, Goel V. Genetic alterations in signaling pathways in melanoma. Clin. Cancer Res. 2006; 12:2301s–2307s. [PubMed: 16609049]

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]

Heider TR, Lyman S, Schoonhoven R, Behrns KE. Ski promotes tumor growth through abrogation of transforming growth factor-beta signaling in pancreatic cancer. Ann. Surg. 2007; 246:61–68. [PubMed: 17592292]

Herlyn M, Shih IM. Interactions of melanocytes and melanoma cells with the microenvironment. Pigment Cell Res. 1994; 7:81–88. [PubMed: 8066024]

Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, Kumar R, Weber BL, Nathanson KL, Phillips DJ, Herlyn M, Schadendorf D, Dummer R. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res. 2006; 19:290–302. [PubMed: 16827748]

Jacob C, Grabner H, Atanasoski S, Suter U. Expression and localization of Ski determine cell type-specific TGFbeta signaling effects on the cell cycle. J. Cell Biol. 2008; 182:519–530. [PubMed: 18695043]

Javelaud D, Alexaki VI, Mauviel A. Transforming growth factor-beta in cutaneous melanoma. Pigment Cell Melanoma Res. 2008; 21:123–132. [PubMed: 18426405]

Kokura K, Kim H, Shinagawa T, Khan MM, Nomura T, Ishii S. The Ski-binding protein C184M negatively regulates tumor growth factor-beta signaling by sequestering the Smad proteins in the cytoplasm. J. Biol. Chem. 2003; 278:20133–20139. [PubMed: 12646588]

Krasagakis K, Garbe C, Orfanos CE. Cytokines in human melanoma cells: synthesis, autocrine stimulation and regulatory functions--an overview. Melanoma Res. 1993; 3:425–433. [PubMed: 8161882]

Krasagakis K, Garbe C, Zouboulis CC, Orfanos CE. Growth control of melanoma cells and melanocytes by cytokines. Recent Results Cancer Res. 1995; 139:169–182. [PubMed: 7597288]

Krasagakis K, Kruger-Krasagakes S, Fimmel S, Eberle J, Tholke D, von der OM, Mansmann U, Orfanos CE. Desensitization of melanoma cells to autocrine TGF-beta isoforms. J. Cell Physiol. 1999; 178:179–187. [PubMed: 10048582]

Le Scolan E, Zhu Q, Wang L, Bandyopadhyay A, Javelaud D, Mauviel A, Sun L, Luo K. Transforming growth factor-beta suppresses the ability of Ski to inhibit tumor metastasis by inducing its degradation. Cancer Res. 2008; 68:3277–3285. [PubMed: 18451154]

Li AG, Lu SL, Zhang MX, Deng C, Wang XJ. Smad3 knockout mice exhibit a resistance to skin chemical carcinogenesis. Cancer Res. 2004; 64:7836–7845. [PubMed: 15520189]

Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC, Shi Y, Chen YG, Meng A, Feng XH. PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. Cell. 2006; 125:915–928. [PubMed: 16751101]

Longerich T, Breuhahn K, Odenthal M, Petmecky K, Schirmacher P. Factors of transforming growth factor beta signalling are co-regulated in human hepatocellular carcinoma. Virchows Arch. 2004; 445:589–596. [PubMed: 15455231]

Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M, Smalley KS, Mahale A, Eroshkin A, Aaronson S, Ronai Z. Rewired ERK-JNK Signaling Pathways in Melanoma. Cancer Cell. 2007; 11:447–460. [PubMed: 17482134]

Luo K, Stroschein SL, Wang W, Chen D, Martens E, Zhou S, Zhou Q. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev. 1999; 13:2196–2206. [PubMed: 10485843]

MacDougall J, Kobayashi H, Kerbel RS. Responsiveness of Normal, Dysplastic Melanocytes, and Melanoma Cells from Different Lesional Stages of Disease Progression to the Growth inhibitory Effects of TGF-beta. Molecular and Cellular Differentiation. 1993; 1:21–40.

Massague J, Gomis RR. The logic of TGFbeta signaling. FEBS Lett. 2006; 580:2811–2820. [PubMed: 16678165]

Matsuzaki K. Smad3 phosphoisoform-mediated signaling during sporadic human colorectal carcinogenesis. Histol. Histopathol. 2006; 21:645–662. [PubMed: 16528675]

Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, Seki T, Taketani S, Fujisawa JI, Okazaki K. Smad2 and Smad3 Phosphorylated at Both Linker and COOH-Terminal Regions Transmit Malignant TGF-{beta} Signal in Later Stages of Human Colorectal Cancer. Cancer Res. 2009

McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC, Lawrence DA. Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. J. Biol. Chem. 2001; 276:33964–33968. [PubMed: 11441025]

Medrano EE. Repression of TGF-beta signaling by the oncogenic protein SKI in human melanomas: consequences for proliferation, survival, and metastasis. Oncogene. 2003; 22:3123–3129. [PubMed: 12793438]

Miyazono K, Suzuki H, Imamura T. Regulation of TGF-beta signaling and its roles in progression of tumors. Cancer Sci. 2003; 94:230–234. [PubMed: 12824914]

Mizuide M, Hara T, Furuya T, Takeda M, Kusanagi K, Inada Y, Mori M, Imamura T, Miyazawa K, Miyazono K. Two short segments of Smad3 are important for specific interaction of Smad3 with c-Ski and SnoN. J. Biol. Chem. 2003; 278:531–536. [PubMed: 12426322]

Muthusamy V, Hobbs C, Nogueira C, Cordon-Cardo C, McKee PH, Chin L, Bosenberg MW. Amplification of CDK4 and MDM2 in malignant melanoma. Genes Chromosomes. Cancer. 2006; 45:447–454. [PubMed: 16419059]

Pardali K, Moustakas A. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim. Biophys. Acta. 2007; 1775:21–62. [PubMed: 16904831]

Reed JA, Bales E, Xu W, Okan NA, Bandyopadhyay D, Medrano EE. Cytoplasmic localization of the oncogenic protein ski in human cutaneous melanomas in vivo: functional implications for transforming growth factor Beta signaling. Cancer Res. 2001; 61:8074–8078. [PubMed: 11719430]

Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. Am. J. Pathol. 1994; 145:97–104. [PubMed: 8030760]

Rodeck U, Bossler A, Graeven U, Fox FE, Nowell PC, Knabbe C, Kari C. Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. Cancer Res. 1994; 54:575–581. [PubMed: 8275496]

Rodeck U, Nishiyama T, Mauviel A. Independent regulation of growth and SMAD-mediated transcription by transforming growth factor beta in human melanoma cells. Cancer Res. 1999; 59:547–550. [PubMed: 9973198]

Sapkota G, Knockaert M, Alarcon C, Montalvo E, Brivanlou AH, Massague J. Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. J. Biol. Chem. 2006; 281:40412–40419. [PubMed: 17085434]

Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res. 2003; 63:756–759. [PubMed: 12591721]

Schmid P, Itin P, Rufli T. In situ analysis of transforming growth factor-beta s (TGF-beta 1, TGF-beta 2, TGF-beta 3), and TGF-beta type II receptor expression in malignant melanoma. Carcinogenesis. 1995; 16:1499–1503. [PubMed: 7614683]

Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, Seki T, Matsui H, Fujisawa J, Okazaki K. Reversible Smad-Dependent Signaling between Tumor Suppression and Oncogenesis. Cancer Res. 2007; 67:5090–5096. [PubMed: 17545585]

Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell. 2004; 117:211–223. [PubMed: 15084259]

Shinagawa T, Nomura T, Colmenares C, Ohira M, Nakagawara A, Ishii S. Increased susceptibility to tumorigenesis of ski-deficient heterozygous mice. Oncogene. 2001; 20:8100–8108. [PubMed: 11781823]

Siegel, PM, Massague, J. Growth Control by TGF-β: Mechanisms Controlling Cell Cycle Progression and Apoptosis. In: Derynck, R, Miyazono, K, editors. The TGF-β Family. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2008. 333–362.

Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA. Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. Mol. Cell. 1999a; 4:499–509. [PubMed: 10549282]

Sun Y, Liu X, Ng-Eaton E, Lodish HF, Weinberg RA. SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. Proc. Natl. Acad. Sci. USA. 1999b; 96:12442–12447. [PubMed: 10535941]

Tabata T, Kokura K, ten DP, Ishii S. Ski co-repressor complexes maintain the basal repressed state of the TGF-beta target gene, SMAD7, via HDAC3 and PRMT5. Genes Cells. 2009; 14:17–28. [PubMed: 19032343]

Takahata M, Inoue Y, Tsuda H, Imoto I, Koinuma D, Hayashi M, Ichikura T, Yamori T, Nagasaki K, Yoshida M, Matsuoka M, Morishita K, Yuki K, Hanyu A, Miyazawa K, Inazawa J, Miyazono K, Imamura T. SKI and MEL1 cooperate to inhibit transforming growth factor-beta signal in gastric cancer cells. J. Biol. Chem. 2009; 284:3334–3344. [PubMed: 19049980]

Teichler S, Schlenk RF, Strauch K, Hagner NM, Ritter M, Neubauer A. Expression of the nuclear oncogene Ski in patients with acute myeloid leukemia treated with all-trans retinoic acid. Haematologica. 2008; 93:1105–1107. [PubMed: 18508800]

Ueki N, Hayman MJ. Direct interaction of Ski with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated repression of transforming growth factor-beta signaling. J. Biol. Chem. 2003; 278:32489–32492. [PubMed: 12857746]

Wang G, Matsuura I, He D, Liu F. Transforming Growth Factor-{beta}-inducible Phosphorylation of Smad3. J. Biol. Chem. 2009; 284:9663–9673. [PubMed: 19218245]

Wang H, Mannava S, Grachtchouk V, Zhuang D, Soengas MS, Gudkov AV, Prochownik EV, Nikiforov MA. c-Myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle. Oncogene. 2008; 27:1905–1915. [PubMed: 17906696]

Watson JD, Oster SK, Shago M, Khosravi F, Penn LZ. Identifying genes regulated in a Myc-dependent manner. J. Biol. Chem. 2002; 277:36921–36930. [PubMed: 12145275]

Wrighton KH, Willis D, Long J, Liu F, Lin X, Feng XH. Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. J. Biol. Chem. 2006; 281:38365–38375. [PubMed: 17035229]

Xu W, Angelis K, Danielpour D, Haddad MM, Bischof O, Campisi J, Stavnezer E, Medrano EE. Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor. Proc. Natl. Acad. Sci. U. S. A. 2000; 97:5924–5929. [PubMed: 10811875]

Yamagata H, Matsuzaki K, Mori S, Yoshida K, Tahashi Y, Furukawa F, Sekimoto G, Watanabe T, Uemura Y, Sakaida N, Yoshioka K, Kamiyama Y, Seki T, Okazaki K. Acceleration of Smad2 and Smad3 phosphorylation via c-Jun NH(2)-terminal kinase during human colorectal carcinogenesis. Cancer Res. 2005; 65:157–165. [PubMed: 15665291]

Yang G, Li Y, Nishimura EK, Xin H, Zhou A, Guo Y, Dong L, Denning MF, Nickoloff BJ, Cui R. Inhibition of PAX3 by TGF-beta modulates melanocyte viability. Mol. Cell. 2008; 32:554–563. [PubMed: 19026785]

Zhang H, Stavnezer E. Ski regulates muscle terminal differentiation by transcriptional activation of Myog in a complex with Six1 and Eya3. J. Biol. Chem. 2008

Zhuang D, Mannava S, Grachtchouk V, Tang WH, Patil S, Wawrzyniak JA, Berman AE, Giordano TJ, Prochownik EV, Soengas MS, Nikiforov MA. C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. Oncogene. 2008; 27:6623–6634. [PubMed: 18679422]

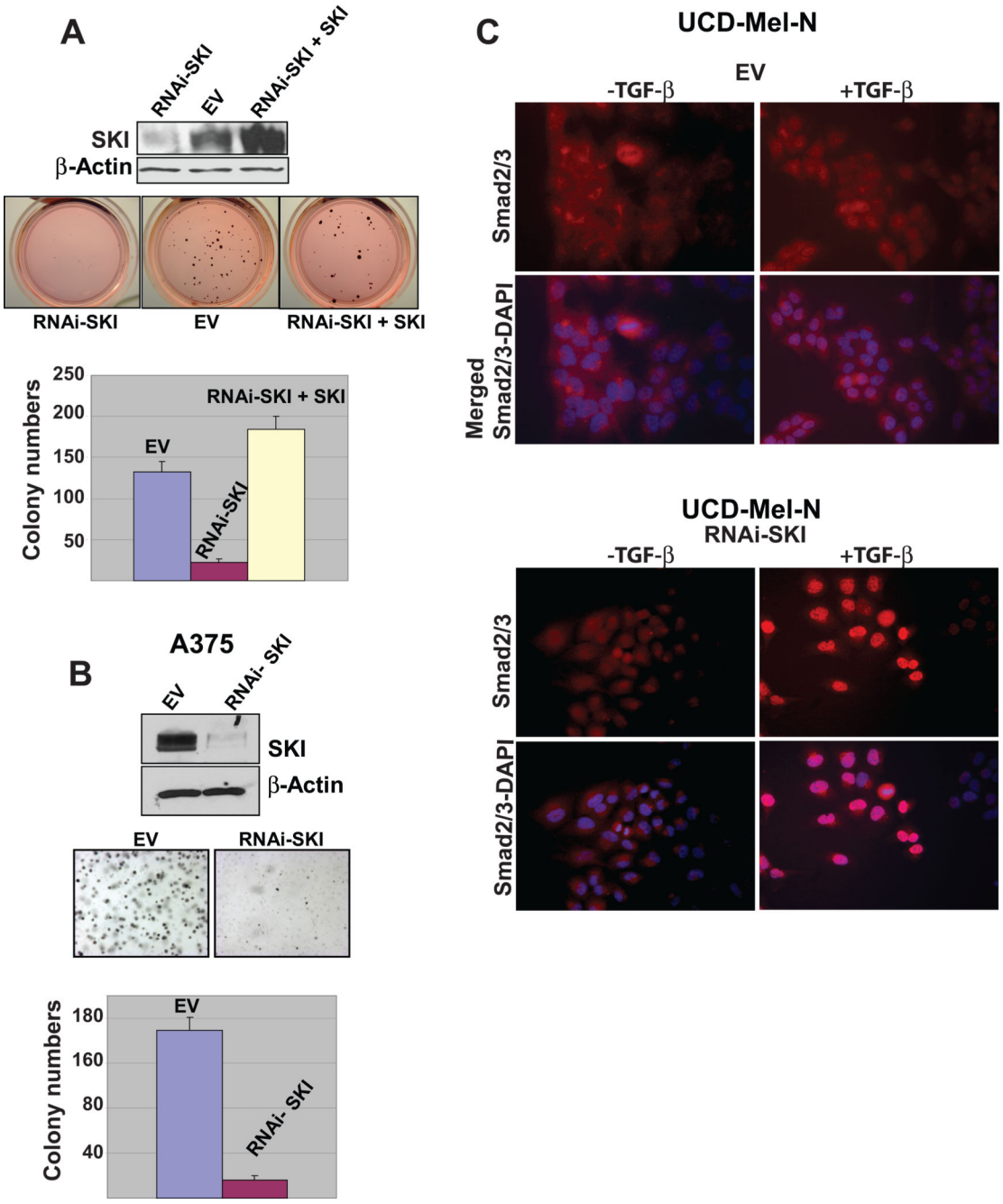

Figure 1. Stable downregulation of endogenous SKI by overexpression of 783-nt SKI ds-RNA impairs anchorage-independent growth in human melanoma cell lines

A) Upper panel: Western blot showing SKI protein levels in pooled clones of UCD-Mel-N expressing empty vector control (EV), RNAi-SKI and SKI transfected into RNAi-SKI cells (rescue experiment). Lower panel: Colony growth in the absence of substratum (agar growth) and quantification of colony inhibition (The mean ± SE of two independent experiments is shown). B) SKI levels in A375 melanoma cells transfected with control (EV) and ds-RNAi-SKI vectors (RNAi-SKI). Colonies were quantified as described above. C) Immunofluorescence analysis showing that down-regulation of SKI significantly enhances

the nuclear localization of Smad2/3 after treatment with 0.6ng/ml TGF-β for 2 hrs (see Material and Methods for details). Smad2/3 proteins were detected by an anti-Smad2/3 Ab and a Texas red-conjugated secondary Ab. DNA was stained by DAPI.

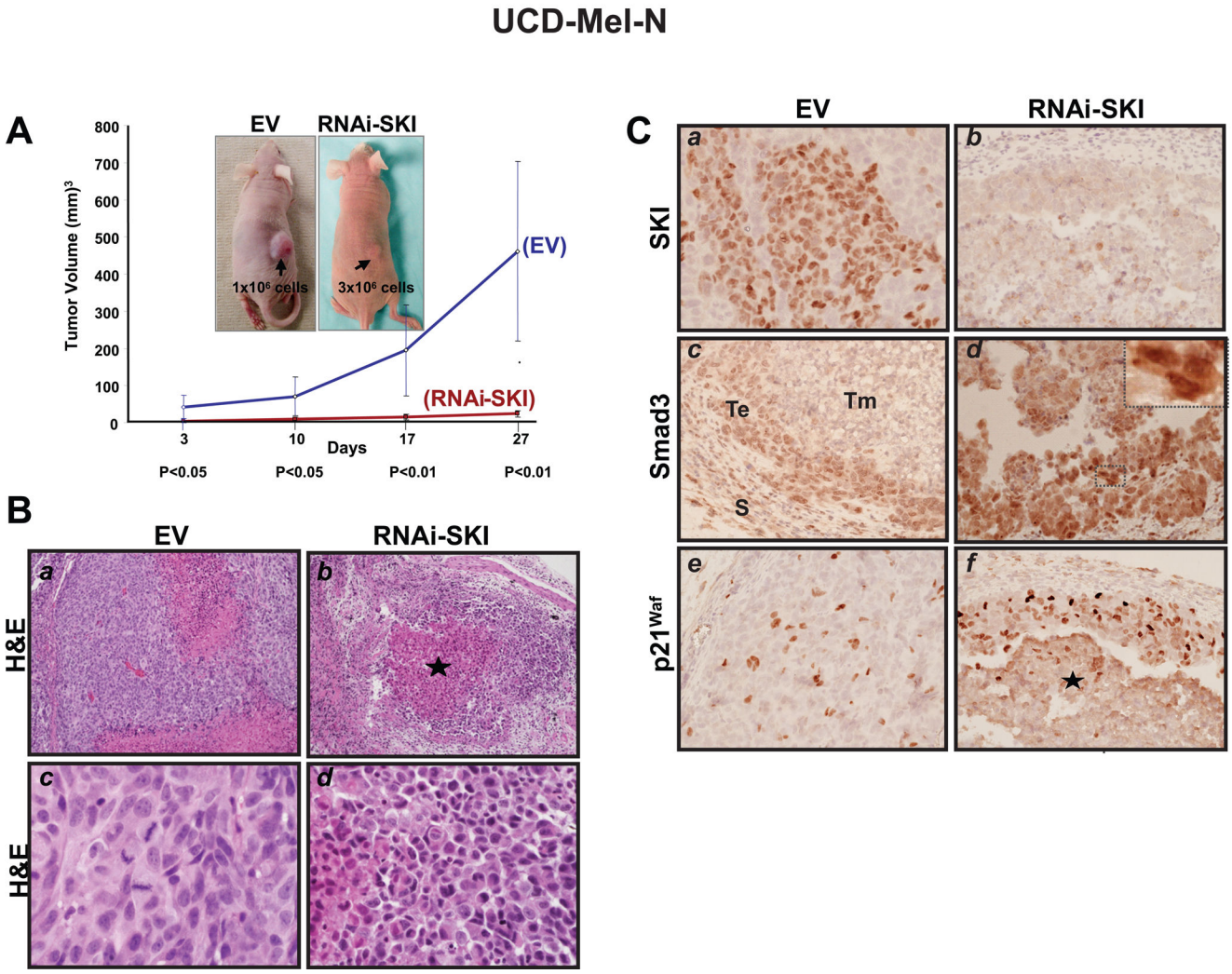

Figure 2. Downregulation of SKI prevents growth of melanoma xenografts by restoring signatures of active TGF-β-growth inhibitory signals

A) Downregulation of SKI generated minimal UCD-RNAi-SKI tumor xenograft growth compared to the robust growth of xenografts displaying endogenous levels of SKI-EV. B) Histopathological assessment of time-matched SKI-EV and RNAi-SKI tumors. Photomicrographs of hematoxylin and eosin (H&E)-stained sections of SKI-EV (left side, panels a, c) show a large tumor nodule with multiple zones of tumor cell necrosis. Tumor cells have a large epithelioid or spindle-shaped appearance and a high mitotic rate. H&E-stained sections of UCD-RNAi-SKI (right side, panels b, d) show a small tumor nodule with prominent central necrosis (star). These tumor cells have a small epithelioid appearance and a low mitotic rate (Supplemental Table 1). C) Immunohistochemical detection of upregulated Smad3 and p21WAF-1 in the small RNAi-SKI knockdown tumors. An anti-SKI antibody confirmed that SKI was still downregulated in the RNAi-SKI tumors compared to SKI-EV controls (top row, panels a, b). Expression of Smad3 (middle row, panels c, d) was seen along the stroma (S) and edges (Te) but not in the middle (Tm) of SKI-EV tumors expressing endogenous SKI compared to the prominent nuclear and cytoplasmic localization

of Smad3 observed in RNAi-SKI tumor cells. Expression of p21WAF-1 (bottom row, panels e, f) was observed in only a few scattered SKI-EV tumor cells compared to the prominent nuclear localization throughout the rim of viable RNAi-SKI tumor cells surrounding zones of necrosis (star). H&E-stained sections, original magnification 33× (top row), 132× (bottom row). Immunohistochemical labeling was performed as described in the Materials and Methods, original magnification 66× (all panels).

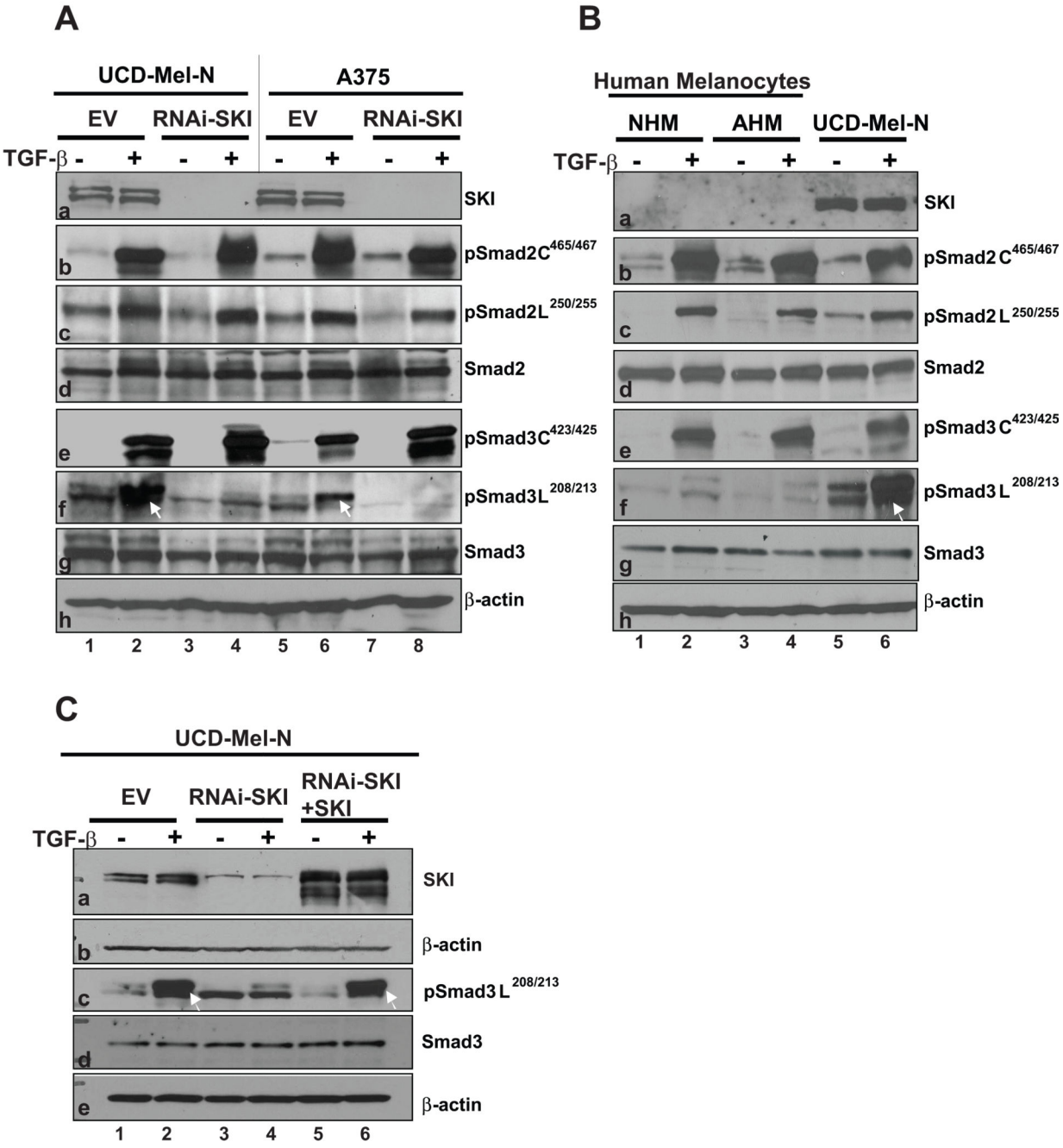

Figure 3. Endogenous SKI is required for Smad3 linker phosphorylations induced by TGF-β in melanoma cells

A) Western blot of total cell extracts using specific anti-pSmad2C (Ser 465–467), anti-pSmad3C (Ser423–425), anti-pSmad2L (S250–255) and anti-Smad3L (S208–213) antibodies. Arrows indicate the dramatic increase in pSmad3L (S208–213) in cells displaying endogenous levels of SKI. B) These amino acid residues are minimally phosphorylated in normal human neonatal (NHM) and adult melanocytes (AHM) compared to the robust phosphorylation induced by TGF-β in wild-type UCD-Mel-N melanoma cells. Cells were treated with 0.6ng/ml TGF-β for 1 hr. C) A Western blot shows that

overexpression of SKI in RNA-SKI UCD-Mel-N melanoma cells (rescue experiment) re-established strong Smad3L phosphorylations (arrows) after TGF-β treatment. Cells were treated with 0.6ng/ml TGF-β for 1 hr.

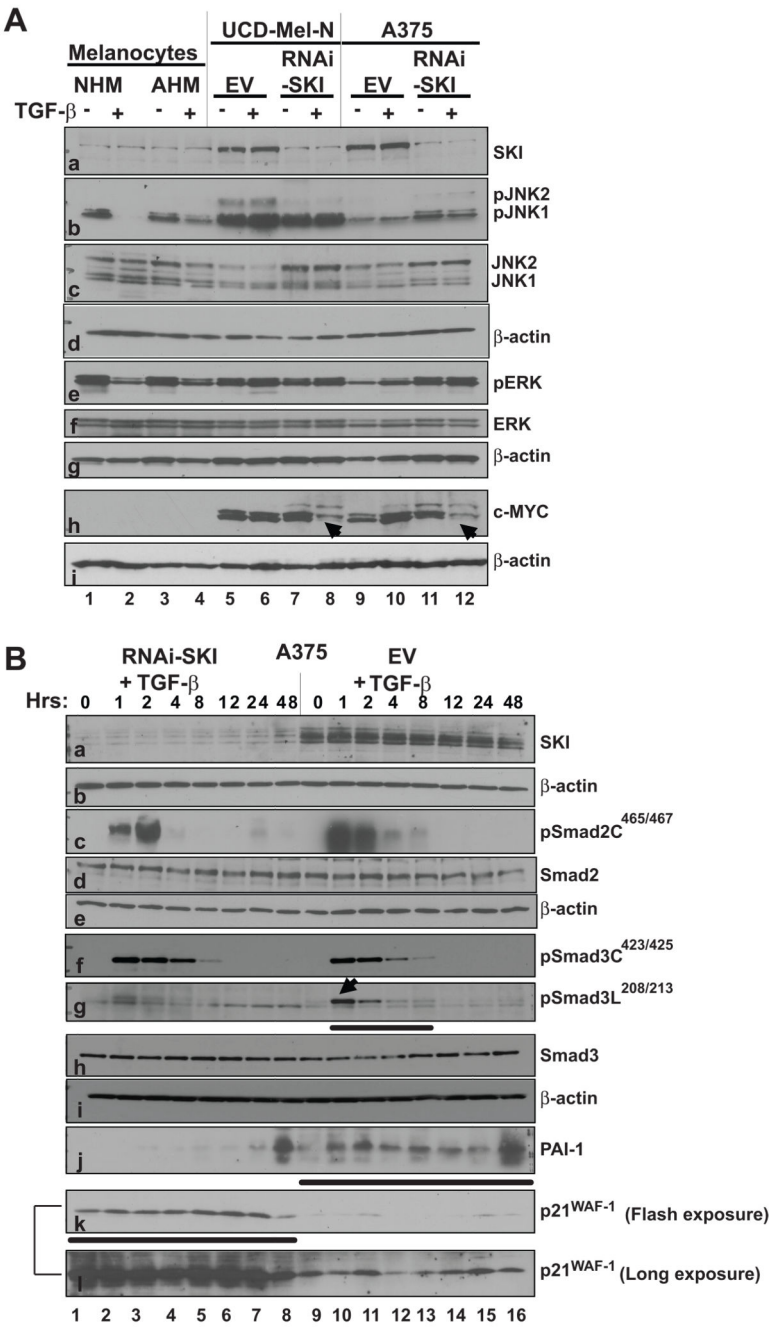

Figure 4. SKI is necessary for preventing TGF-β-mediated down-regulation of c-MYC and for up-regulation of PAI-1

A) Levels of p-ERK, pJNK and c-MYC in normal melanocytes, UCD-Mel-N and A375 melanoma cell lines and derivatives expressing RNA-SKI. Protein extracts (50 μg/ml) were analyzed by western blots with antibodies indicated in the figure. The arrowheads indicate downregulation of c-MYC in RNAi-SKI melanoma cells. Cells were treated with 0.6ng/ml TGF-β for 1 hr. B) Western blot of total cell extracts prepared after TGF-β treatment. The arrow indicates robust pSmad3L phosphorylation that peaked at 2 hrs after TGF-β treatment. Black lines indicate significant changes in pSmad3L phosphorylation, PAI-1 and $p21^{Waf-1}$

expression between RNAI-SKI and EV control cells. Cells were treated with 0.6ng/ml TGF-β for the times indicated in the figure.

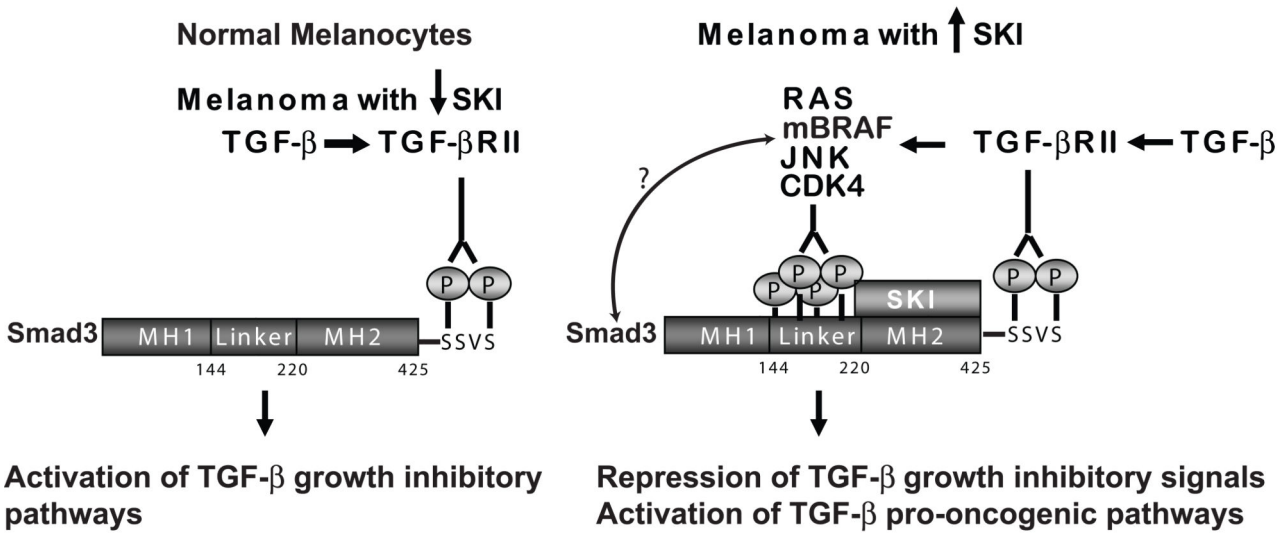

Figure 5. A working model suggesting that SKI stimulates the pro-oncogenic pathway of TGF-β in melanoma cells (data modified from (Matsuzaki, 2006))

TGFβ binds TGF-βRII, which in turns recruits the low affinity receptor (TGF-βRI) to activate the pathway (Groppe et al., 2008). Complex cross-talks between TGF-β, RAS, JNK, CDK2, and CDK4, a protein overexpressed in a subset of melanomas displaying amplification of the *CDK4* gene (Muthusamy et al., 2006), result in Smad3 phosphorylations at Thr^{179} and Ser^{208} (Matsuzaki et al., 2009; Wang et al., 2009). The model suggests that by enhancing Smad3L phosphorylations, SKI becomes a major regulator of the switch of TGF-β from a growth inhibitor to a pro-oncogenic protein in human malignant melanoma.