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REVIEW

Ski and SnoN, potent negative regulators of TGF-β signaling

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Ski and the closely related SnoN were discovered as oncogenes by their ability to transform chicken embryo fibroblasts upon overexpression. While elevated expressions of Ski and SnoN have also been reported in many human cancer cells and tissues, consistent with their pro-oncogenic activity, emerging evidence also suggests a potential anti-oncogenic activity for both. In addition, Ski and SnoN have been implicated in regulation of cell differentiation, especially in the muscle and neuronal lineages. Multiple cellular partners of Ski and SnoN have been identified in an effort to understand the molecular mechanisms underlying the complex roles of Ski and SnoN. In this review, we summarize recent findings on the biological functions of Ski and SnoN, their mechanisms of action and how their levels of expression are regulated.

***Keywords*:** SnoN, Ski, Signal transduction, development, tumorigenesis, TGF-β signaling, senescence
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Introduction

The *sno* gene (Ski novel gene) was initially discovered on the basis of its close homology to v-*ski*, the transforming component of the Sloan–Kettering virus (SKV) [1] and its cellular homolog c-*ski* [2]. The human Ski and SnoN proteins share an overall 50% amino acid (aa) homology (and 36% identity) with similar domain structures (Figure 1). The full-length *sno* gene encodes the 684-amino-acids SnoN protein as well as some reported alternatively spliced forms. These include SnoN2 (or mSno-dE3), resulting from a 46-aa deletion in exon 3, SnoI, which contains only the first 399 residues of SnoN, and SnoA, which differs from SnoN after exon 1 with a specific and shortened C-terminal end (Figure 1) [2-5]. The *ski* gene has not been shown to be regulated by alternative splicing.

Ski and SnoN are members of the Ski family that also contains two recently described members: Fussel-18 (Functional Smad suppressing element on chromosome 18) [6] and Fussel-15 (or LBXCOR1) [7]. Both Fussel-18 and Fussel-15 have significant homology with Ski and SnoN, and can interact with R-Smads in transfected cells and inhibit TGF-β or BMP responsive gene expression in reporter assays. Unlike Ski and SnoN, the expression of Fussel-18 and Fussel-15 appears to be limited to neuronal tissues, suggesting that their functions may be more restricted.

Ski and SnoN contain several structural domains (Figure 1), including the DHD (Dachshund homology domain, also called DS domain for DACH, Ski and Sno) in their N-terminal region [8, 9] and a Smad4 binding domain [10]. The 100-aa DHD forms a compact, globular structure consisting of mixed alpha helix and beta sheets [11] that has features found in the forkhead/winged-helix family of DNA-binding proteins. Since neither Ski nor SnoN has been shown to directly bind DNA, the DHD may function to mediate interaction of Ski/SnoN with other proteins, such as the transcriptional co-regulator N-CoR (nuclear receptor co-repressor) [12]. Close to the DHD domain stands an 80-aa C2H2 Zn-binding module with structural homology to the SAND domain (Sp100, AIRE1, NucP41/75 and DEAF1) [10]. This domain is only present in Ski, SnoN and Fussels, and is necessary for interacting with Smad4 [10]. The C-terminal regions of Ski and SnoN are less conserved, but may allow Ski and SnoN to homo- or hetero-dimerize through a coiled-coil motif located in this region [13, 14].

Biological functions of Ski and SnoN

In embryonic development

The *ski* and *sno* genes, although highly homologous, are not functionally redundant during development. *In*

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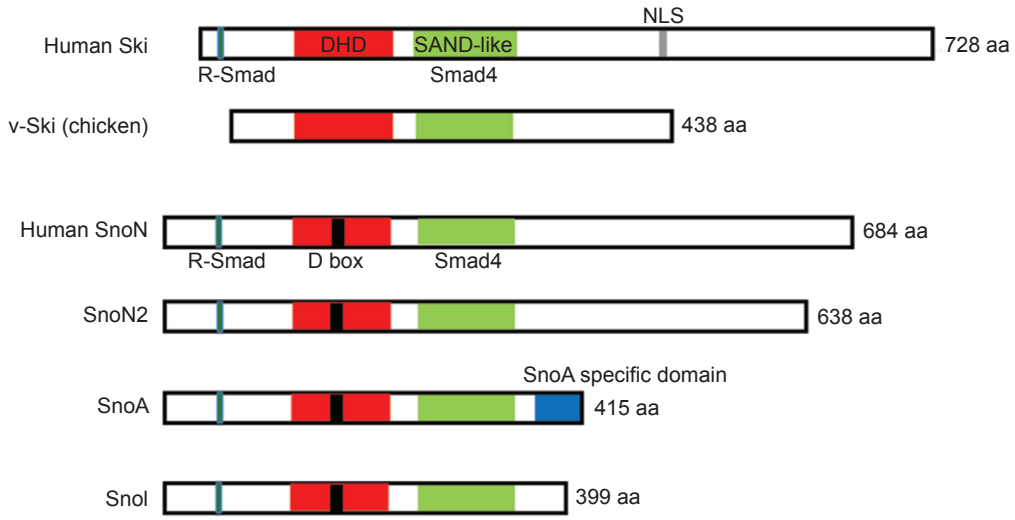

Figure 1 Domain structure of Ski and SnoN family of proteins. The number of amino acids for each protein is indicated on the right. DHD: Dachshund homology domain. SAND: Sp100, AIRE1, NucP41/75 and DEAF1. NLS: Nuclear localization sequence. D box: Degradation box. Explanations for domain functions are given in the main text.

vivo studies in *Xenopus* oocytes, zebrafish and mice have demonstrated a critical role of *ski* in the development of neuronal and muscle lineages [15-17]. In *Xenopus*, the XSki protein is maternal, and is maintained at a high level until the late neurula stage [18]. When injected into the *Xenopus* embryos, Ski was found to induce secondary neural axis formation and neural specific gene expression in ectoderm explants [17]. In mouse, Ski is expressed in all normal adult and in embryonic tissues at a low level [19]. Its expression is increased from E8.5 to E9.5 and from E12.5 to E15.5 as well as in some tumor cells in the adult. The first peak of Ski expression during embryogenesis (E8.5-9.5) correlates with neural tube closure and migration of neural crest cells, and the second peak overlaps with skeletal muscle development (E12.5-E15.5). This expression pattern indicates that Ski may play a role in the development of muscle, craniofacial structure, central and peripheral nervous systems, and respiratory tissues in the mouse embryo [20]. Supporting this notion, *ski*-null pups suffer from exencephaly, the absence of the cranial vault, due to failed neural tube closure and die perinatally [16]. In addition, these fetuses display facial clefting of varying severity and other craniofacial and skeletal abnormalities. The *ski*-null mice also show a marked decrease in skeletal muscle mass, probably due to a defect in secondary myoblast proliferation and/or survival [16]. The human *ski* gene is located on chromosome 1p36.3, and monosomy of this region of chromosome 1 results in the 1p36 syndrome, a genetic disorder characterized by craniofacial defects including a tower skull with a large and late closing anterior fontanel and a prominent forehead [21]. Interestingly, the phenotypes of the *ski*-knockout mice mirror many symptoms of the human 1p36 syndrome [22], suggesting that deletion of the *ski* gene may be partially responsible for the disease. Three lines of *sno*-knockout mice have been generated and published. One knockout mouse was shown to die before implantation [23], while two other mice with deletion of either the first exon of the *sno* gene (thus lacking both the DHD and the Smad4 binding domain) or the *sno* promoter are viable without apparent developmental defects [24]. Since the cause of this discrepancy has not been determined, the function of SnoN in embryonic development remains unclear.

In adult tissues

Ski and SnoN are expressed in virtually all adult tissues, albeit at low levels [2, 3, 9]. They can regulate the differentiation of several cell types, particularly those in neural and muscle lineages. In chicken and quail embryo fibroblasts, overexpression of either WT Ski or SnoN induces muscle differentiation [25, 26]. In mice, loss of *ski* leads to reduced skeletal muscle mass [16, 22], whereas Ski overexpression causes muscle hypertrophy [27]. Consistent with these results, Ski and SnoN have been shown to activate the expression of muscle-specific genes [28, 29]. In a tissue culture setting, SnoN also regulates neuronal cell function, for example as a potent promoter of axonal growth [30]. Similarly, Ski has been implicated in regulation of Schwann cell proliferation and myelina-

tion [31]. In addition to muscle and neuronal lineages, Ski has also been shown to influence the growth and differentiation of hematopoietic cells *in vitro* [32, 33], and SnoN may be involved in regulation of T-cell function as two of the sno gene knockout mouse strains display defective T-cell activation [24]. Finally, the expression of Ski and SnoN is altered under many pathological conditions, including wound healing [34], liver regeneration [35] and obstructive nephropathy [36, 37]. The biological significance of these alterations is not clear. Although Ski and SnoN are expressed in all epithelial cells, their functions in normal epithelial cells have not been defined.

In cancer progression

The original discovery of Ski as the cellular homolog of a viral transforming protein leads to the classification of c-*ski* as a proto-oncogene. Indeed, early studies show that overexpression of Ski or SnoN promotes oncogenic transformation of avian fibroblasts as well as myogenic differentiation of quail embryo cells [25, 26, 38]. These early studies highlight an apparent contradiction, as Ski or SnoN overexpression favors either transformation or terminal differentiation, and hint at the potential complexity of Ski/SnoN action.

Supporting the pro-oncogenic properties of Ski and SnoN, expression of Ski and/or SnoN is elevated in many cancer cells and tissues, including those derived from esophageal squamous cell carcinoma [39-41], melanoma [42-44], estrogen receptor-positive breast carcinoma [45], colorectal carcinoma [46] and leukemia [33, 47]. Moreover, the human *sno* gene is located at 3q26.2, a chromosome locus frequently amplified in many tumors, including cancers of the lung [48, 49], esophagus [39], head and neck [50, 51], cervix [49, 52], ovary [49, 53] and prostate [54]. However, this chromosomal localization by itself is not sufficient to suggest a pro-oncogenic role of SnoN as the locus also contains many other genes that are known to promote cancer progression. The strongest evidence supporting the pro-oncogenic function of Ski or SnoN in mammalian tumorigenesis comes from the studies showing that reduction of SnoN expression by siRNA in human lung or breast cancer cells inhibits tumor growth both *in vitro* and *in vivo* [55], and that downregulation of Ski in pancreatic cancer cells also reduces tumor growth [56]. However, decreasing Ski expression by siRNA in lung and breast cancer cells has no effect on the transforming activity of these cells [55], suggesting that the tumor-promoting activity of Ski may

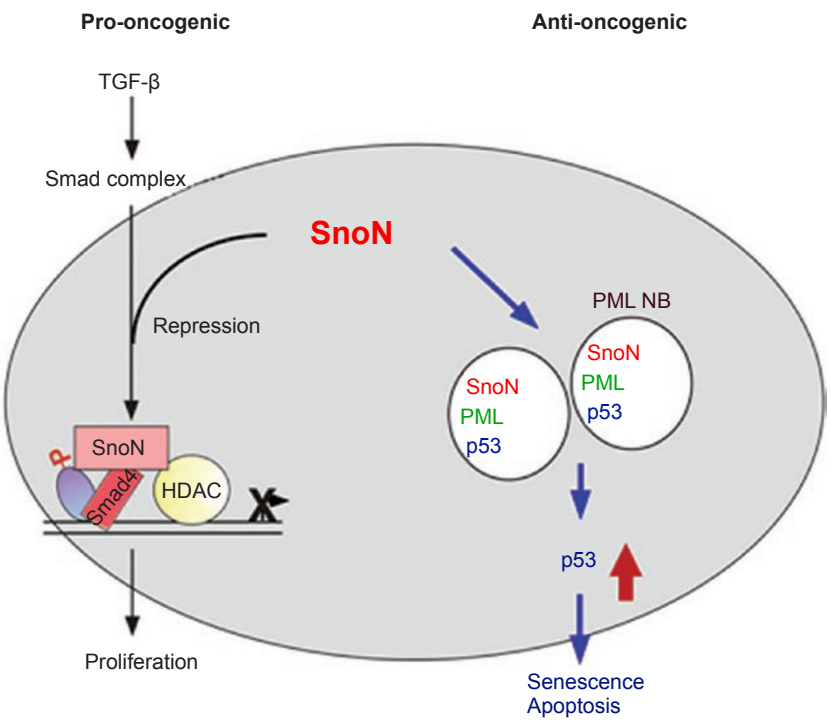

Figure 2 SnoN possesses both pro-oncogenic and anti-oncogenic activities. In the nucleus, SnoN can have either pro-oncogenic or anti-oncogenic activities through two pathways. SnoN may promote epithelial cell proliferation by antagonizing the growth-inhibitory activity of the TGF-β/Smad pathway. Very high levels of SnoN may also trigger premature senescence through stabilizing p53 in a PML-dependent manner, and this pathway mediates the anti-oncogenic activity of SnoN.

be restricted to certain types of cancers.

More recent surveys in human cancer tissues reveal a more complex pattern of Ski and SnoN expression during malignant progression. In tissue samples from patients with Barrett's esophagus, Ski and SnoN were found to be expressed at high levels in tissues with low grade dysplasia but absent in those with high grade dysplasia or adenocarcinoma [57]. In colorectal cancer with microsatellite instability, SnoN is downregulated in 39% of the samples and upregulated in 33% of the samples [58]. These results imply that high levels of Ski and SnoN may not always be beneficial to cancer cells. Indeed in recent years, more evidence has emerged indicating that both Ski and SnoN can act as tumor suppressors. First, heterozygous *sno*$^{+/-}$[23] and *ski*$^{+/-}$ [59] mice are more susceptible to carcinogen-induced tumors than WT mice, suggesting that an extra copy of the *ski* or *sno* gene protects against carcinogenesis *in vivo*. Recently, we showed that high levels of SnoN induce premature senescence through stabilization of p53. High levels of SnoN increase the transcription of PML, and through a physical interaction with the increased PML protein, SnoN is in turn recruited to the PML nuclear bodies, leading to stabilization of p53 (Figure 2). Consistent with a tumor suppressor function of senescence, overexpression of SnoN inhibits oncogene-induced transformation of primary MEF cells. More importantly, in a two-step skin carcinogenesis model, mice expressing a high level of SnoN were resistant to the development of papilloma and displayed senescence *in vivo*. This activity of SnoN is independent of its ability to antagonize the Smad proteins and provides a mechanistic basis for the tumor suppressor function of SnoN (Figure 2) [60]. Consistent with this tumor suppressor activity, the Drosophila dSno protein also inhibits cell growth [61]. Finally, downregulation of Ski or SnoN by shRNA enhances EMT of lung and breast cancer cells *in vitro* and tumor metastasis *in vivo* [55, 62]. These data highlight the complex nature of Ski/SnoN functions in cancer, with both pro- and anti-oncogenic properties.

Molecular mechanisms of Ski and SnoN action

Ski and SnoN do not have catalytic activities, and therefore have to function through interaction with other cellular partners. Their primary function is related to transcriptional regulation, despite that they are unable to bind DNA directly. They can interact with a number of transcription factors, including Smads [63, 64], pRb [65], GATA1 [66], Gli3 [67] or retinoic acid receptor α (RARα) [32, 47], and transcriptional co-regulators such as N-CoR [68] or mSin3A [12].

Negative regulation of TGF-β signaling

So far the most important function described for Ski and SnoN is to negatively regulate TGF-β signaling by binding to the Smad proteins [69]. Ski and SnoN interact simultaneously with the R-Smad (Smad2/3), through their N-terminal region, and with co-Smad (Smad4), through the SAND-like domain [10], and block the ability of the Smad complexes to activate transcription of TGF-β target genes. A comparison of the crystal structure of the Smad4 binding domain of Ski in complex with Smad4 with that of the phospho-Smad2/Smad4 complex showed that Ski and phospho-Smad2 compete for binding to the same region on Smad4, predicting that binding of Ski to Smad4 results in the displacement of phospho-Smad2/3 from Smad4 and disruption of the functional Smad heteromeric complexes [10]. Indeed, biochemical analysis using Ski truncation mutants defective in binding to one of the Smad proteins confirmed this model [10]. Although both the R-Smads and Smad4 remain bound to Ski, they are no longer in an active heteromeric complex that could activate transcription [70]. Moreover, binding of Ski or SnoN may additionally stabilize the inactive Smad heteromer on DNA, possibly preventing further binding of active Smad complexes [70]. A later study showed that Smad3 could still be found in complex with Smad4 in the presence of a mutant Ski defective in binding to Smad3, arguing that the Smad complex may be inactivated instead of being disrupted [71, 72]. However, since not all the Smad complexes in the cells are bound to Ski, the detected Smad complex described above could belong to the Ski-independent population of Smads. Moreover, the above interactions were detected under overexpression conditions, and it is not clear whether this holds true under physiological conditions of Smad expression. In addition to disruption of the active Smad complexes, Ski or SnoN also prevents binding of the R-Smads to transcriptional co-activator p300/CBP [10, 73] and actively recruits a transcriptional co-repressor complex containing N-CoR and HDAC to the targeted promoters [63, 64]. It is likely that all these mechanisms function together to allow antagonism of Smad signaling by Ski/SnoN. High levels of Ski have also been reported to interfere with Smad2/3 phosphorylation by TβRI [74]. Since Ski and SnoN are primarily located in the nucleus, any potential effect on receptor-mediated phosphorylation of Smad2/3 is unlikely to be direct.

Since Smads are critical mediators of the growth inhibitory signals of TGF-β in epithelial cells, high levels of Ski or SnoN may promote cell proliferation through inhibiting the Smad proteins. Indeed, many human cancer cell lines express high levels of Ski or SnoN and are refractory to TGF-β-induced growth arrest [42, 55, 75].

Reducing Ski and/or SnoN expression in these cancer cells restores TGF-β signaling and renders these cells sensitive to the growth inhibitory effects of TGF-β. These results suggest that the transforming activity of Ski or SnoN may be related to their ability to antagonize the Smads. Indeed, we have shown that at least in chicken embryo fibroblast cells where the oncogenic activity of Ski and SnoN was first defined, the ability of Ski and SnoN to bind to and repress the Smad proteins is required for their transforming activity [76]. In other pathological situations such as liver damage and regeneration where the growth inhibitory activity of TGF-β needs to be suppressed, SnoN is found to be upregulated [35].

The TGF-β superfamily of proteins, in particular BMP and activin play important roles in multiple aspects of vertebrate development. As Ski and SnoN also play important roles in embryonic development and in the differentiation of multiple cell lineages, an obvious question is whether these activities of Ski and SnoN are dependent on their ability to repress BMP or activin signaling. Interestingly, Ski, but not SnoN, can bind to and repress the activity of BMP-specific Smads, Smad1/5 [77, 78]. In *Xenopus* embryos, this ability of Ski to repress BMP signaling is required for the specification of neural cell fate [78]. Moreover, many craniofacial phenotypes found in the *ski*-null mice are reminiscent of those found in transgenic mice overexpressing a BMP target gene Msx2, or in embryos in which expression of Msx2 had been activated by BMP2 and BMP4 [79, 80]. Thus, it is possible that the balance of Ski and BMP4 activity may be critical for normal craniofacial and muscle development and that the craniofacial abnormalities found in *ski*-null mice are due to lack of repression of BMP signaling.

Apart from the antagonistic activity of SnoN on TGF-β signaling, one recent study suggested surprisingly that at very low concentrations SnoN may also serve as a cell type-specific co-activator of TGF-β signaling in a mink lung epithelial cell line [75]; and this activity may be mediated by the interaction with ING2 [81]. Whether this is true for other cell lines and the biological relevance of these findings remain to be determined.

Stabilization of p53 by SnoN through binding to PML

Our recent study has revealed a novel functional pathway of SnoN in regulation of p53 expression (Figure 2). SnoN can interact with the PML protein through residues 322-366 immediately following the Smad4 binding domain and is recruited to the PML nuclear bodies. This results in stabilization of p53 and premature senescence [60]. The activity of SnoN to induce senescence is independent of its ability to bind to the Smad proteins and antagonize TGF-β signaling but is associated with elevated SnoN expression. Mutant SnoN that is defective in binding to the Smad proteins associates with PML, stabilizes p53 and induces senescence as efficiently as WT SnoN. These data reveal a Smad-independent function of SnoN and provide a mechanism for SnoN function as a tumor suppressor.

Other partners of Ski and SnoN

Other cellular partners of Ski generally fall into two major categories. The first group includes members of the transcriptional repressor complex. Ski and SnoN can interact with N-CoR and silencing mediator of retinoid and thyroid hormone receptors (SMRT), members of HDAC complexes [12, 68, 82], and bridge the repressor complex to Smads [63, 64], thyroid hormone receptor and Mad [12], or Gli3 [67] to mediate transcriptional repression by these proteins. Transcriptional repression by Mad may also involve a physical interaction between Ski and PML [83]. In transfected cells, Ski but not SnoN has also been reported to interact with mSin3A, a general co-repressor involved in HDAC complex recruitment [12], HIPK2 (homeodomain-interacting protein kinase 2), another co-repressor necessary for Ski-dependent negative regulation of BMP-induced transcription [84], and MeCP2 (Methyl CpG binding protein 2) [85]. However, these interactions have not been shown with endogenous proteins, and whether they play a role in transcriptional repression by Ski is not clear.

The second group of Ski-binding partners tends to be molecules involved in cell differentiation, proliferation or hormonal responses. Ski has been shown to interact with RARα to block its transactivation activity [32, 47], with PU.1 to negatively regulate macrophage differentiation [86], with GATA1 to block its DNA-binding activity [66], with vitamin D receptor to repress vitamin D signaling [82] and with pRb to abrogate Rb-mediated transcriptional repression [65]. Recently, Ski has been found to be required for TGF-β-induced proliferation of Schwann cells, possibly by inactivating Rb through promoting hyperphosphorylation and cytoplasmic translocation of Rb [87].

Ski has been shown to activate myogenic differentiation by modulating transcriptional activity of the MyoD complex at the myogenin promoter and by inhibiting HDAC activity [28]. This mechanism may be related to the ability of Ski to promote muscle development *in vivo*. Finally, Ski can activate Wnt/β-catenin signaling in melanoma cells by interacting with FHL2 [44]. Another transcriptional activator, SKIP (Ski interacting protein), has been reported to bind to Ski and antagonize its transcriptional co-repressor activity [88], thereby releasing molecules targeted by Ski, such as Smads or pRb [89,

90]. It is worth noting that all the interactions mentioned above are described in transfected cells under overexpression conditions. They have yet to be confirmed *in vivo* and their biological significance under physiological conditions has yet to be determined.

Some of the Ski partners described above have not been shown to bind to SnoN. Similarly, not all the events regulated by SnoN are shared by Ski. For example, SnoN but not Ski has been reported to co-operate with p53 to repress transcription from the alpha-fetoprotein (AFP) promoter [91]. SnoN but not Ski has been shown to inhibit the RhoA small GTPase activity [55]. Thus, Ski and SnoN do not necessarily perform redundant functions in cells.

Regulation of Ski and SnoN expression

As both overexpression and downregulation of Ski or SnoN have been linked to cancer progression, the expression levels of both proteins need to be tightly controlled. Regulation of Ski and SnoN expression occurs at multiple levels, including transcriptional regulation, protein degradation, post-translational modifications and subcellular localization.

Transcriptional regulation

The best studied inducer of SnoN transcription is TGF-β. SnoN transcription is upregulated after 2 h of TGF-β treatment through a direct binding of the Smad2/Smad4 complex to the Smad-binding elements (SBE) in the *sno* promoter [63]. This upregulation of SnoN expression may function as a negative feedback mechanism to turn off TGF-β signaling or may perform other functions including promoting transformation of fibroblasts by TGF-β [92]. Hepatocyte Growth Factor/Scatter Factor (HGF/SF) can upregulate SnoN expression in proximal tubular kidney epithelial cells (HKC-8) [93, 94], but not in glomerular mesangial cells and interstitial fibroblasts [94, 95], indicating that HGF/SF may be a cell type-specific activator of SnoN expression. This activation appears to involve a concerted binding of CREB and Sp1 to their cognate *cis*-acting elements in the *sno* promoter in response to HGF/SF stimulation [94]. Upregulation of SnoN expression has also been observed during liver regeneration [35], possibly mediated by HGF/SF.

In contrast to SnoN, Ski has not been shown to be a direct transcriptional target of TGF-β signaling. In one recent report, low-dose TGF-β (25 pg/mL) was found to induce a slight increase in Ski level in skin fibroblasts, but high-dose TGF-β (25 ng/mL) repressed it [96]. It is not clear whether this regulation occurs at the level of transcription or protein stability and whether this is a direct effect of TGF-β signaling.

Protein degradation

Both SnoN and Ski are regulated at the level of protein stability. Earlier experiments showed that SnoN has a half-life of approximately 4 h in the absence of stimuli, and within 30 min of TGF-β stimulation this is shortened to around 45 min [63, 97]. Shortly after, SnoN was found to be targeted by the ubiquitin-dependent proteasome. Several E3 ubiquitin ligases have been shown to be recruited to SnoN for its ubiquitination in response to TGF-β, including Smurf2 (Smad ubiquitin regulatory factor), a HECT domain-containing E3 ubiquitin ligase [98], the Anaphase Promoting Complex (APC/C), a RING finger ubiquitin ligase complex [99-101], and Arkadia, a RING domain-containing E3 ligase [102, 103]. These molecules interact with the R-Smads, Smad2 and Smad3, and are recruited to SnoN to promote its ubiquitination and degradation. Stable interaction of SnoN with the APC/C complex also requires the Destruction box (D box), present in the DHD of SnoN, that is recognized and bound by CDH1, the targeting subunit of APC/C. Three lysine residues in the C-terminal domain of SnoN, K440, K446 and K449, have been shown to be necessary for SnoN ubiquitination [100]. As these 3 residues are located in the exon that is not present in SnoN2, SnoN2 could be a natural variant of SnoN that is resistant to ubiquitin-dependent degradation.

Given that SnoN is expressed in virtually all cell types, the rapid degradation of SnoN in response to TGF-β stimulation releases its repression of the Smad heteromeric complexes and allows activation of TGF-β target genes. In human esophageal cancer cells, a truncated SnoN, which cannot be degraded accumulates to a high level in cells, resulting in resistance to TGF-β-induced growth arrest [104]. This finding highlights the importance of SnoN stability in regulating cell transformation. Ubiquitin-dependent degradation of SnoN may also play important roles in other physiological and pathological processes. For instance, Smad2-dependent degradation of SnoN through the CDH1/APC/C pathway inhibits axonal growth [30, 105]. Regulation of SnoN expression in obstructive nephropathy has been shown to also involve the ubiquitin-proteasome pathway [36, 37].

Unlike SnoN, TGF-β-induced degradation of Ski is not observed in all cell types. An earlier study showed that Ski is degraded in response to TGF-β in mink lung epithelial cells [97]. However this was not observed in primary ovarian epithelial cells or ovarian cancer cell lines [106]. We recently showed that in metastatic breast cancer and melanoma cell lines, Ski is effectively degraded by TGF-β signaling via Arkadia, which interacts

with Ski through Smad2 and Smad3 to mediate its ubiquitination and degradation [62, 102]. Since As TGF-β is a potent promoter of breast cancer and melanoma metastasis, and Ski can inhibit breast cancer metastasis [55], this degradation of Ski induced by TGF-β may play an important role in malignant progression of these cancer cells. So far, the lysine residues necessary for ubiquitination have not been mapped in Ski, and those lysines targeted by ubiquitination in SnoN are not conserved in Ski. Despite the fact that Ski also contains the D Box motif, the CDH1-APC complex has not been shown to bind to and downregulate Ski.

It has been observed that the expression of SnoN and Ski is regulated throughout the cell cycle. Although some data suggest that SnoN expression is higher in G1 [3, 29], others argue that the relative level of Ski and SnoN is higher in G2/M [99, 107, 108]. The cellular machinery responsible for the cell cycle-dependent regulation of these two proteins has not been identified although it has been suggested that the APC/C complex may be responsible for modulating SnoN expression [99], while cdc34 may regulate Ski expression [107] during the cell cycle independent of TGF-β signaling.

Other post-translational modifications

SnoN can be modified by SUMOylation on lysines 50 and 383 through the action of the SUMO E3 ligase PIASs in a manner independent of TGF-β signaling [109, 110]. SUMOylation of SnoN is independent of its ubiquitination and does not alter its stability or subcellular localization, but may potentiate SnoN-mediated repression of TGF-β signaling on specific promoters [110]. Importantly, mutant SnoN, which cannot be SUMOylated potently activates muscle-specific gene expression and enhances myotube formation. Therefore, SUMOylation of SnoN may block its myogenic activity [109, 110] by an unknown mechanism. Ski has not been reported to be modified by SUMOylation, and the two key lysine residues important for SnoN SUMOylation are not conserved in Ski.

Both Ski and SnoN have been reported to be phosphorylated. SnoN can be phosphorylated on several residues including S115, S117 and/or T119 by TAK1 (TGF-β-activated kinase) [111]. This phosphorylation may facilitate ubiquitination and degradation of SnoN. Interestingly, these residues are not conserved in Ski. Ski is found to be phosphorylated on serine residues in transfected cells [112] and can serve as a substrate of the CDC2 kinase in an *in vitro* kinase assay [108]. However, neither the sites of phosphorylation in Ski nor the kinases responsible for the phosphorylation have been determined. The physiological significance of Ski phosphorylation is not clear.

Subcellular localization

Both Ski and SnoN were identified as nuclear proteins. However, more recent work suggests that they can localize to the cytoplasm under various conditions. In normal tissues and non-tumorigenic or primary epithelial cells, SnoN is predominantly expressed in the cytoplasm, while in malignant tissues and cell lines, it is exclusively nuclear [113]. The cytoplasmic SnoN is resistant to TGF-β-induced degradation and inhibits TGF-β signaling through sequestration of the Smads in the cytoplasm. In mammary epithelial cells, SnoN can translocate to the nucleus upon morphological differentiation or cell cycle arrest. Two lysine residues, Lys30 and Lys31, have been shown to be necessary for SnoN nuclear localization. These residues may be subjected to post-translational modifications or be involved in interaction with yet-to-be-identified partners that regulate SnoN nuclear import/export. Ski has been reported to be predominantly localized in the nucleus [38], while several reports described cytoplasmic localization of Ski in malignant tumor cells [40, 114]. A nuclear localization sequence has been mapped in Ski (residues 452-458) that is not conserved in SnoN [115]. Cytoplasmic Ski inhibits Smad activity but does not activate the myogenin promoter, suggesting that subcellular localization of Ski differentially regulates its activity [115]. A cytoplasmic protein, C184M, has been shown to sequester Ski in the cytoplasm when overexpressed [116]. However, whether this protein is required for the cytoplasmic localization of Ski in metastatic cancer cells *in vivo* is not clear. Given that their nuclear localization sequences are not shared and conserved, it is likely that subcellular localization of Ski and SnoN is regulated through different mechanisms.

Conclusion and future directions

Studies in the past few years have uncovered mechanisms that regulate Ski and SnoN expression at the level of transcription, protein stability or intracellular localization, and have defined the interaction between SnoN/Ski and the Smads through structural and functional studies. Many interacting partners of Ski and SnoN have been identified. As we learn more about their functions, the complex roles of Ski and SnoN in regulation of mammalian epithelial cell function, transformation and vertebrate development are beginning to be revealed. In mammalian tumorigenesis, both pro-oncogenic and anti-oncogenic activities have been appreciated for these two proteins. Some but not all of these activities are dependent on the interaction between Ski/SnoN and the Smad proteins. A goal in the near future is to identify and characterize Smad-independent intracellular pathways

that may be targeted by Ski/SnoN. While much attention in the past has been devoted to revealing the roles of Ski/SnoN in human cancer cells, little is known about their functions in normal mammalian epithelial cells. This is a major challenge that deserves more research efforts in the future.

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