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Identification of a phosphorylation site in c-Ski as serine 515

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Motoko Nagata[1,2], Shinji Nagata[3], Keiko Yuki[1], Kazunobu Isogaya[1], Masao Saitoh[1,4], Kohei Miyazono[1] and Keiji Miyazawa[1,4,*]

[1]Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033; [2]Kasai R & D Center, Daiichi Sankyo Co. Ltd, Tokyo 134-8630; [3]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-0032; and [4]Department of Biochemistry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan

*Keiji Miyazawa, Department of Biochemistry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110, Shimokato, Chuo, Yamanashi 409-3898, Japan. Tel/Fax: 81 55 273 6784, email: keiji-miyazawa@umin.ac.jp

c-Ski has been known to be phosphorylated at serine residue(s), which results in slower migration of c-Ski in SDS–polyacrylamide gel electrophoresis. The position(s) of phosphorylation, however, has not been determined. In the present study, we identified a phosphorylation site of c-Ski which affects its electrophoretic motility as serine 515 using MALDI–TOF mass spectrometry. A phosphorylation-resistant mutant, c-Ski S515A, did not exhibit a phosphatase-sensitive band shift. In addition, we confirmed that endogenous c-Ski is phosphorylated at serine 515, using a specific antibody. The phosphorylation status of c-Ski, however, does not appear to affect its stability or effects on TGF-β signalling. Identification of the phosphorylation site of c-Ski would allow us further examination of physiological significance of c-Ski phosphorylation.

Keywords: c-Ski/oncogene/phosphorylation/Smad/TGF-β.

Abbreviation: TGF-β, transforming growth factor-β.

c-Ski was originally identified as a cellular counterpart of a retroviral oncogene product, v-Ski (*1*). Overexpression of v-Ski as well as c-Ski induces transformation in chicken and quail embryonic fibroblasts (*2*), and also induces muscle differentiation in quail embryonic epiblasts (*3*). c-Ski has been reported to be highly expressed in tumour cell lines and in melanomas, oesophageal and colorectal carcinomas *in vivo* (*4–6*).

Recently, biochemical analyses revealed the role of c-Ski in the signal transduction of transforming growth factor (TGF)-β family members. c-Ski physically interacts with Smad2, Smad3 and Smad4, thus antagonizing signal transduction in the TGF-β pathway (*7–10*) as well as the bone morphogenetic protein pathway (*11*, *12*). Inhibition of cytostatic function of TGF-β is considered to be a part of the mechanism of oncogenesis due to c-Ski (*13*).

Phosphorylation of c-Ski was first reported by Sutrave *et al.* (*14*). They compared characteristics of the full-length chicken c-Ski with those of its truncated isoforms, and found that the full-length c-Ski is serine-phosphorylated whereas C-terminally truncated forms are not. Recently, Marcelain and Hayman (*15*) reported that phosphorylated c-Ski migrates more slowly on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) than does unphosphorylated c-Ski. However, the phosphorylation site(s) in c-Ski as well as the physiological significance of the phosphorylation remains to be determined.

In the present study, we identified a phosphorylation site of c-Ski which affects its electrophoretic motility as serine 515. We confirmed that endogenous c-Ski is phosphorylated at the site using an immunochemical approach. We further examined alterations in biochemical characteristics of c-Ski upon phosphorylation at serine 515, including its stability and effects on TGF-β signalling.

Materials and Methods

Cell culture

HEK293T, COS7, HCT116, HepG2 and HeLa cells were obtained from American Type Culture Collection. HEK293T, COS7, HepG2 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) and HCT116 cells were cultured in RPMI1640 medium (Sigma), supplemented with 10% foetal bovine serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin).

Plasmid constructions

c-Ski and c-Ski mutants ΔA, ΔB, ΔC and ΔD were described earlier (*7*). Other c-Ski mutants were generated using a PCR-based approach.

Antibodies

Anti-c-Ski antibody used for immunoprecipitation was described earlier (*16*). Anti-c-Ski-pS515 was prepared as follows: a peptide CKDLGS(PO_4)PGARA, which corresponds to the sequence surrounding the phosphorylated site of c-Ski (residues 510–520), was synthesized and conjugated to keyhole limpet haemocyanin (Calbiochem) using succinimidyl 4-(*p*-maleimidophenyl) butyrate (Pierce). The conjugate was then injected into rabbits in Freund's complete adjuvant and anti-sera were collected. The antibody, anti-c-Ski-pS515, was purified by two steps of column chromatography using an Affigel-10 (Bio-Rad) coupled with CKDLGS(PO_4)PGARA and *o*-phosphoserine-agarose (Sigma) as described earlier (*17*). Anti-c-Ski (G8, Cascade BioScience), anti-phosphoserine (Poly-Z-PS-1; #-61-8100, Zymed), anti-FLAG (M2, Sigma) antibodies were used as primary antibodies in immunoblotting.

Transfection, immunoprecipitation and immunoblotting

Cells were transfected using FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's recommendations. Cell lysates were prepared in a buffer containing 20 mM Tris–HCl

(pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1.5% Trasylol (Bayer) and 1 mM phenylmethlsulfonyl fluoride. After clearing with centrifugation, supernatants were subjected to immunoprecipitation or immunoblotting as described earlier (*16*). Some of the samples were treated with 5 U of calf intestine alkaline phosphatase (ALP, TOYOBO) at 30°C for 15 min or 200 U of λ protein phosphatase (PPase, New England BioLabs) at 30°C for 30 min after immunoprecipitaion. $NaVO_3$ was added to the reaction mixture at 1 mM (final concentration).

Mass spectrometric measurement

Coomassie Brilliant Blue-stained bands in the electrophoresed gel were excised and in-gel digested by sequence-grade trypsin (Roche) [2 μg in 20 μl of 100 mM $(NH_4)_2CO_3$] at 37°C overnight. The solution containing the peptide fragments was subjected to Zip-Tip C18 (Millipore) and the peptide fragments were eluted by 60% acetonitrile/0.1% trifluoroacetic acid after washing the Tip with 0.1% trifluoroacetic acid. Molecular weights of the peptide fragments prepared by in-gel digestion procedure was analysed by MALDI–TOF MS (Voyager-DE STR, Applied Biosystems), performed by accelerating voltage at 20.0 kV of the reflector mode. The supernatant of the saturated solution of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile/0.1% trifluoroacetic acid was used as a matrix solution (the matrix solution:sample containing peptides = 1 : 1). The measurement of molecular weights was calibrated with the peptide fragments derived from the self-digest of trypsin.

siRNA

The siRNA for human c-Ski (SKI Stealth Select RNAi; HSS109772) was purchased from Invitrogen and was transfected to cells using HiPerfect (QIAGEN) according to the manufacturer's recommendations.

Pulse-chase analysis and luciferase reporter assay

Pulse-chase analysis and luciferase reporter assay were performed as described earlier (*16*).

Results and Discussion

Phosphorylation of c-Ski results in slower migration in SDS–PAGE

It has been shown that c-Ski protein exhibits doublet bands in transfected cells. The band shift corresponded to approximately 8,000 Da increase in its apparent molecular weight. Recently, it has been reported that the upper band disappears upon treatment with phosphatase (*15*). When we performed pulse-chase analysis of transfected c-Ski protein, the upper band was negligible just after pulse, but increased during chase period (Fig. 1A). These finding indicate that the upper band corresponds to post-translationally modified c-Ski. We also found that the upper band reacted with anti-phosphoserine antibody (Fig. 1B). Treatment with phosphatase resulted in disappearance of the reactivity as well as the band shift, both of which were not observed when phosphatase activity was inhibited with sodium pervanadate. These findings, together with the previous report (*15*), indicate that the upper band corresponds to phosphorylated c-Ski. The band shift was not observed in a c-Ski homologous protein SnoN (data not shown).

Mapping of the phosphorylated region in c-Ski using truncated mutants

We next tried to map the phosphorylation site(s) that are related to the band shift by analysis using truncated mutants of c-Ski. c-Ski wt and four truncated mutants ΔA–ΔD (Fig. 2A) were expressed in 293T cells, and

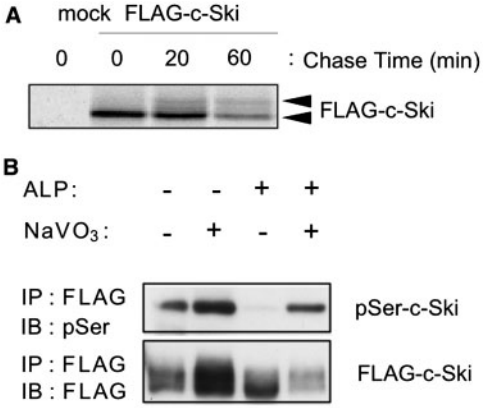

Fig. 1 Confirmation of the phosphorylation of c-Ski. (A) Post-translational modification of c-Ski detected in pulse-chase analysis. Transfected COS7 cells were labelled with [^{35}S]-methionine and cysteine for 10 min, and chased for indicated period of time. c-Ski protein was immunoprecipitated from cell lysates using anti-FLAG antibody and analysed. (B) Detection of phosphorylation of c-Ski in the upper band using anti-phosphoserine antibody. c-Ski was immunoprecipitated from transfected COS7 cells with anti-FLAG antibody, and analysed by immunoblotting using anti-phophoserine as well as anti-FLAG antibodies. Some of the samples were treated with ALP and/or sodium vanadate ($NaVO_3$) after immunoprecipitaion.

the band shift was examined (Fig. 2B). A mutant with C-terminal truncation, ΔD that corresponds to residues 1–490 of c-Ski, migrated as a single band, whereas a mutant with N-terminal truncation, ΔB that corresponds to residues 491–728, exhibited doublet bands, suggesting that the modified residue(s) is located in ΔB. Consistent with these observations, ΔA (residues 338–728) exhibited the band shift, but ΔC (residues 338–490) did not. We then narrowed down the phosphorylation site(s) using three truncated mutants, ΔB499 that corresponds to residues 499–728, ΔB505 that corresponds to residues 505–728 and ΔB520 that corresponds to residues 520–728 (Fig. 2C). The mutant proteins ΔB499 and ΔB505 as well as ΔB exhibited the band shift, whereas ΔB520 did not (Fig. 2D). These results suggest that the phosphorylation site(s) related to the band shift reside residues between 505 and 520. This region, however, still contains five serine residues (Fig. 2C).

Identification of the phosphorylation site of c-Ski as serine 515

To identify the phosphorylated residue(s), the mutant ΔB was overexpressed in HEK293T cells and separated on SDS–PAGE. The upper as well as the lower band was excised from the gel, trypsin-digested and analysed on MALDI–TOF mass spectrometry. The samples were divided into two portions; one half was directly analysed and another half was analysed after phosphatase treatment. Phosphorylated fragments are known to be difficult to detect because of the low efficiency of ionization (*18*). As shown in Fig. 3A, a peak at m/z 772.4 was detected in the upper band only after phosphatase treatment, while it was detected in the lower band irrespective to phosphatase treatment. These findings suggest that this peptide fragment

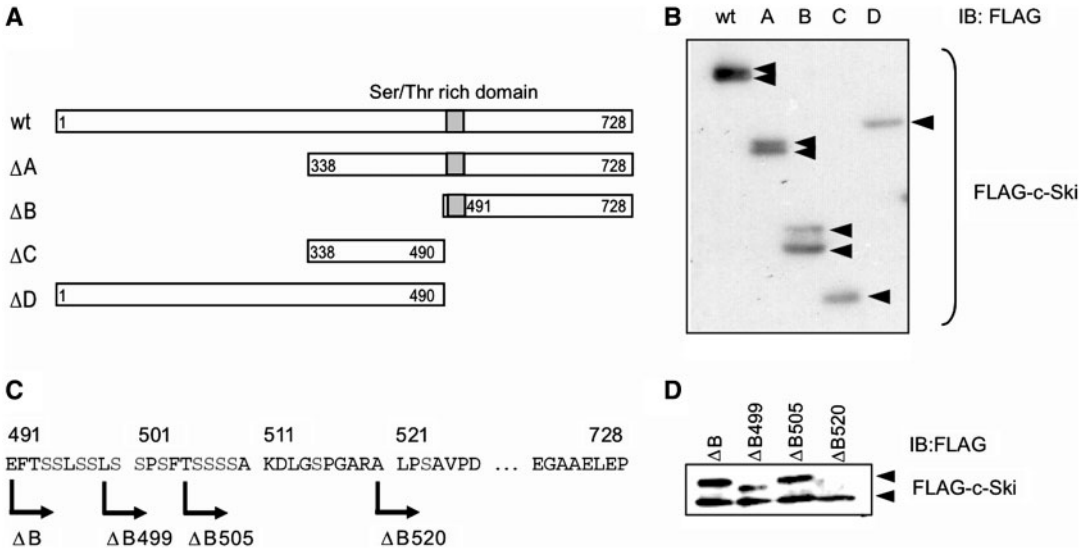

Fig. 2 Mapping of the phosphorylation site that is responsible for band shift of c-Ski using truncated mutants. (A) Schematic presentation of c-Ski truncated mutants ΔA–ΔD. (B) Electrophoretic properties of c-Ski truncated mutants (ΔA-ΔD) in SDS–PAGE (10%). (C) N-terminal amino acid sequences of c-Ski ΔB-derived mutants. (D) Electrophoretic properties of truncated mutants derived from ΔB in SDS–PAGE.

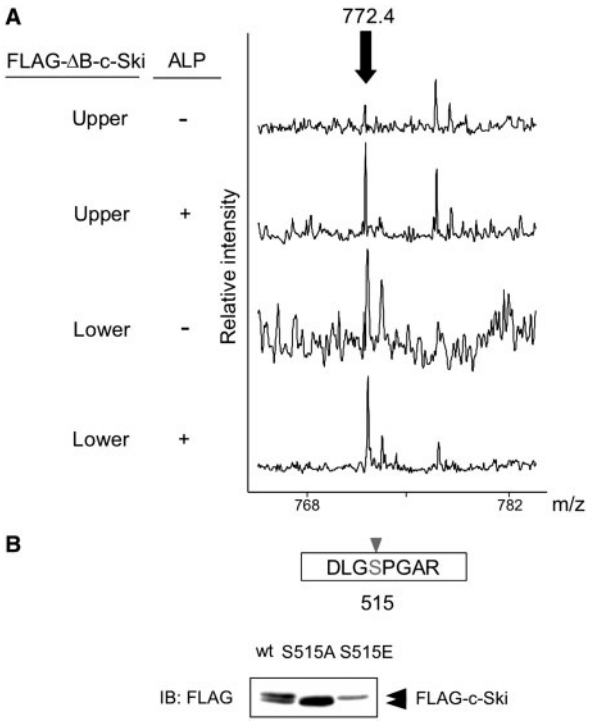

Fig. 3 Identification of the major phosphorylation site of c-Ski as serine 515. (A) Identification of the phosphorylated residues in c-Ski ΔB using MALDI–TOF MS analysis. c-Ski ΔB was transiently expressed in HEK293T cells and separated on SDS–PAGE. After staining with Coomassie Brilliant Blue, the upper as well as the lower band were excised from the gel and trypsin-digested. The samples were then divided into two portions; one half was directly analysed and another half was analysed after phosphatase treatment. (B) Electrophoretic mobility of c-Ski-S515A and -S515E mutant proteins. FLAG-tagged mutants of c-Ski were expressed in HEK293T cells and separated on SDS–PAGE (7.5%), followed by immunoblotting detection using anti-FLAG antibody.

contains phosphorylated residue(s) responsible for the band shift. From its molecular mass, the fragment appeared to correspond to DLGSPGAR (Fig. 3A, residues 512–519 of c-Ski). Serine residue at 515 is thus likely to be phosphorylated in the upper band, which is consistent with the results obtained from c-Ski deletion mutants (Fig. 2D). We then constructed expression plasmids with mutation of serine 515 in c-Ski to alanine (c-Ski-S515A), and found that the mutant migrated as a single band that corresponds to the lower band (Fig. 3B). In contrast, c-Ski-S515E, a mutant that mimics phosphorylation, migrated more slowly (Fig. 3B). We concluded that S515 of c-Ski is a phosphorylated site to cause the band shift.

Confirmation of endogenous c-Ski phosphorylated at serine 515 by an immunological technique

Phosphorylation of S515 in c-Ski was further confirmed by an immunological technique. Antibody was raised against a phosphopepetide KDLGS(PO_4)PGARA, which corresponds to the region surrounding S515 of c-Ski. The anti-serum exclusively reacted with the upper band of c-Ski wt, but with neither the lower band nor c-Ski-S515A (Fig. 4A). In addition, the reactivity to the upper band was sensitive to phosphatase treatment (Fig. 4B). These findings suggest that the anti-serum specifically reacts to c-Ski that is phosphorylated at S515 (c-Ski-pS515). Anti-c-Ski-pS515 antibody was then affinity-purified for further analyses.

We next examined whether the phosphorylation at S515 occurs in endogenous c-Ski protein. Human colorectal carcinoma HCT116 cells were used because the cells express a high level of c-Ski protein. c-Ski was immunoprecipitated, followed by immunoblotting using antibodies recognizing total c-Ski or c-Ski-pS515 (Fig. 4C). Multiple bands corresponding to 81, 78, 73, 68 and 66 kDa were detected by immunoblotting using anti-c-Ski. These bands disappeared upon knockdown of c-Ski by siRNA, confirming that these are c-Ski gene products. Among these, two bands corresponding to 78 and 68 kDa were detected using anti-c-Ski-pS515. These bands also disappeared upon knockdown

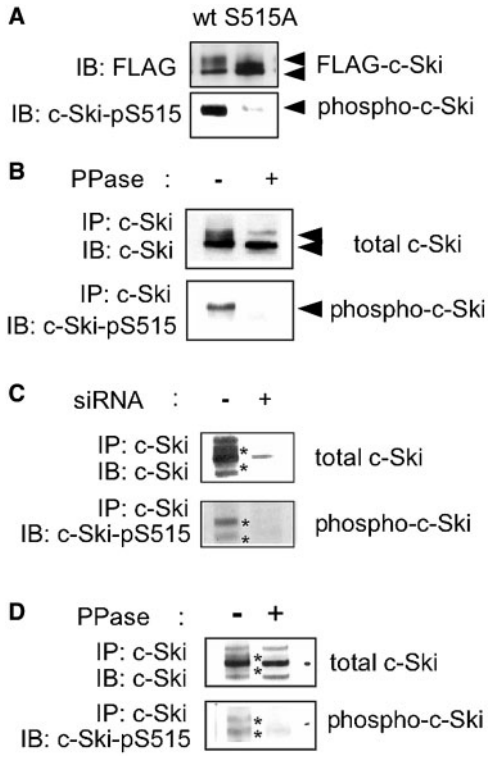

Fig. 4 Phosphorylation of endogenous c-Ski at serine 515. (A) Anti-c-Ski-pS515 anti-serum did not react with c-Ski-S515A mutant protein. FLAG-tagged c-Ski WT or c-Ski-S515A was expressed in HEK293T cells and subjected to immunoblotting analysis using anti-FLAG antibody or anti-c-Ski-pS515 anti-serum. (B) Anti-c-Ski-pS515 anti-serum did not react with dephosphorylated c-Ski protein. c-Ski was expressed in HEK293T cells and subjected to immunoblotting analysis after treatment with λPPase. (C) and (D) Detection of endogenous c-Ski phosphorylated at serine 515. Endogenous c-Ski was immunoprecipitated from cell lysates prepared from HCT116 cells using anti-c-Ski antibody, followed by immunoblotting with anti-c-Ski (G8) or anti-c-Ski-pS515. Specificity of the reactivity was confirmed by knockdown using siRNA or phosphatase treatment.

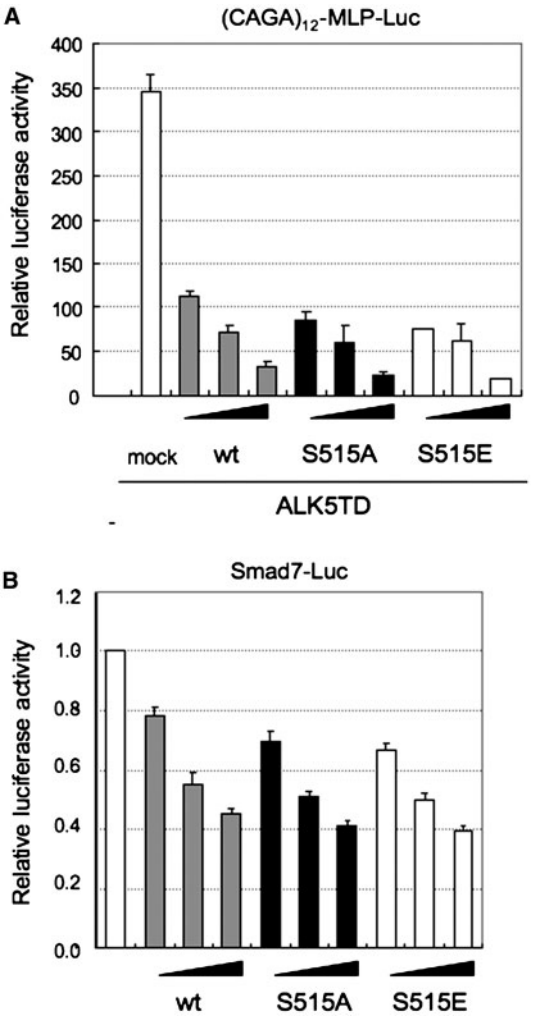

Fig. 5 Effects of c-Ski S515 mutants on c-Ski-regulated transcriptional activity. Luciferase reporter assay was conducted in HepG2 cells using $(CAGA)_{12}$-MLP-Luc in the presence of TGF-β signalling (A) or Smad7-Luc (B). ALK5TD is a constitutively active mutant of TGF-β type I receptor.

of c-Ski by siRNA, or phosphatase treatment (Fig. 4C and D), thus validating the specificity of the antibody. We concluded that endogenous c-Ski is phosphorylated at S515.

Biochemical properties of c-Ski mutants S515A and S515E

We finally examined whether phosphorylation of c-Ski at S515 affects its biochemical properties. c-Ski has been known to potently suppress TGF-β signalling (*7–10*). We thus examined effects of c-Ski-S515A and -S515E on TGF-β-induced transactivation of $(CAGA)_{12}$-MLP-Luc reporter. These mutants, however, inhibited TGF-β signalling to a similar extent as c-Ski wt (Fig. 5A). c-Ski has recently been reported to inhibit basal Smad7 gene expression through Smad4-mediated binding to the Smad7 promoter region (*19*). We examined effects of c-Ski S515 mutants on basal activity of Smad7-Luc (*16*), but they inhibited the reporter activity similarly to c-Ski wt (Fig. 5B). Phosphorylation at S515 does not appear to affect activities of c-Ski which are related to Smad signalling.

The possible relationship between phosphorylation of c-Ski and its stabilization has been suggested, although devoid of direct demonstrations. Sutrave *et al.* (*14*) observed that truncated c-Ski mutants that are not phosphorylated are less stable than the wild-type protein. Marcelain and Hayman (*15*) reported that protein expression level of c-Ski increases during mitosis, which is accompanied by an increase in a slower mobility form of c-Ski due to its phosphorylation. We thus examined stability of c-Ski-S515A as well as -S515E by pulse chase analysis in transfected COS7 cells, because phosphorylation at S515 results in slower motility in SDS–PAGE. These mutants exhibited similar stability compared with c-Ski wt (data not shown), excluding the role of c-Ski phosphorylation at this site in its stabilization. Recently, Band *et al.* reported that phosphorylation of c-Ski at T458 by Akt results in destabilization of c-Ski through proteasomal degradation (*20*).

The physiological significance of phosphorylation of c-Ski at S515 thus remains to be determined.

Our present study, however, would help further understanding of physiological as well as pathological roles of c-Ski.

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Conflict of interest

None declared.

References

1. Li, Y., Turck, C.M., Teumer, J.K., and Stavnezer, E. (1986) Unique sequence, ski, in Sloan-Kettering avian retroviruses with properties of a new cell-derived oncogene. *J. Virol.* **57**, 1065–1072
2. Colmenares, C., Sutrave, P., Hughes, S.H., and Stavnezer, E. (1991) Activation of *c-ski* oncogene by overexpression. *J. Virol.* **65**, 4929–4935
3. Colmenares, C. and Stavnezer, E. (1989) The *ski* oncogene induces muscle differentiation in quail embryo cells. *Cell* **59**, 293–303
4. Reed, J.A., Bales, E., Xu, W., Okan, N.A., Bandyopadhyay, D., and Medrano, E.E. (2001) Cytoplasmic localization of the oncogenic protein Ski in human cutaneous melanomas in vivo: functional implications for transforming growth factor β signaling. *Cancer Res.* **61**, 8074–8078
5. Fukuchi, M., Nakajima, M., Fukai, Y., Miyazaki, T., Masuda, N., Sohda, M., Manda, R., Tsukada, K., Kato, H., and Kuwano, H. (2004) Increased expression of c-Ski as a co-repressor in transforming growth factor-β signaling correlates with progression of esophageal squamous cell carcinoma. *Int. J. Cancer* **108**, 818–824
6. Buess, M., Terracciano, L., Reuter, J., Ballabeni, P., Boulay, J.-L., Laffer, U., Metzger, U., Herrmann, R., and Rochlitz, C.F. (2004) Amplification of *SKI* is a prognostic marker in early colorectal cancer. *Neoplasia* **6**, 207–212
7. Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., and Kawabata, M. (1999) c-Ski transcriptional co-repressor in transforming growth factor-β signaling through interaction with Smads. *J. Biol. Chem.* **274**, 35269–35277
8. Luo, K., Stroschein, S.L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) The Ski oncoprotein interacts with the Smad proteins to repress TGF-β signaling. *Genes Dev.* **13**, 2196–2206
9. Sun, Y., Liu, X., Eaton, E.N., Lane, W.S., Lodish, H.F., and Weinberg, R.A. (1999) Interaction of the Ski oncoprotein with Smad3 regulates TGF-β signaling. *Mol. Cell* **4**, 499–509
10. Xu, W., Angelis, K., Danielpour, D., Haddad, M.M., Bischof, O., Campisi, J., Stavnezer, E., and Medrano, E.E. (2000) Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type β transforming growth factor. *Proc. Natl Acad. Sci. USA* **97**, 5924–5929
11. Wang, W., Mariani, F.V., Harland, R.M., and Luo, K. (2000) Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. *Proc. Natl Acad. Sci. USA* **97**, 14394–14399
12. Takeda, M., Mizuide, M., Oka, M., Watabe, T., Inoue, H., Suzuki, H., Fujita, T., Imamura, T., Miyazono, K., and Miyazawa, K. (2004) Interaction with Smad4 is indispensable for suppression of BMP signaling by c-Ski. *Mol. Biol. Cell* **15**, 963–972
13. He, J., Tegen, A.B., Krawitz, A.R., Martin, G.S., and Luo, K. (2003) The transforming activity of Ski and SnoN is dependent on their ability to repress the activity of Smad proteins. *J. Biol. Chem.* **278**, 30540–30547
14. Sutrave, P., Copeland, T.D., Showalter, S.D., and Hughes, S.H. (1990) Characterization of chicken *c-ski* oncogene products expressed by retrovirus vectors. *Mol. Cell. Biol.* **10**, 3137–3144
15. Marcelain, K. and Hayman, M.J. (2005) The Ski oncoprotein is upregulated and localized at the centrosomes and mitotic spindle during mitosis. *Oncogene* **24**, 4321–4329
16. Nagata, M., Goto, K., Ehata, S., Kobayashi, N., Saitoh, M., Miyoshi, H., Imamura, T., Miyazawa, K., and Miyazono, K. (2006) Nuclear and cytoplasmic c-Ski differently modulate cellular functions. *Genes Cells* **11**, 1267–1280
17. Inoue, T., Kataoka, H., Goto, K., Nagaike, K., Igami, K., Naka, D., Kitamura, N., and Miyazawa, K. (2004) Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci.* **95**, 803–808
18. McLachlin, D.T. and Chait, B.T. (2001) Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* **5**, 591–602
19. Denissova, N.G. and Liu, F. (2004) Repression of endogenous *Smad7* by Ski. *J. Biol. Chem.* **279**, 28143–28148
20. Band, A.M., Bjorklund, M., and Laiho, M. (2009) The phosphatidylinositol 3-kinase/Akt pathway regulates transforming growth factor-β signaling by destabiliszing Ski and inducing Smad7. *J. Biol. Chem.* **284**, 35441–35449