Ski is involved in transcriptional regulation by the repressor and full-length forms of Gli3

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Transcription factor Glioblastoma-3 (Gli3) is cleaved in the anterior region of the limb bud to generate its repressor form. In contrast, Sonic hedgehog (Shh) signaling from the posterior zone of polarizing activity blocks Gli3 processing and then induces the expression of Gli3 target genes, including Gli1. Here we report that the Ski corepressor binds to Gli3 and recruits the histone deacetylase complex. The Gli3-mediated repression was impaired by anti-Ski antibody and in Ski-deficient fibroblasts, and Shh-induced Gli1 gene transcription mediated by full-length Gli3 was inhibited by Ski. Furthermore, a Ski mutation enhanced the digit abnormalities caused by the Gli3 gene mutation. Thus, Ski plays an important role in pattern formation.

Supplemental material is available at http://www.genesdev.org.

Received June 21, 2002; revised version accepted September 23, 2002.

In Drosophila, a transcription factor Cubitus interruptus (Ci) mediates Hedgehog (Hh) signaling [Alexandre et al. 1996, Domiguez et al. 1996]. In the absence of Hh signaling, Ci is processed into a repressor, whereas Hh signaling prevents this Ci cleavage, generating a full-length Ci activator [Aza-Blanc et al. 1997]. In mice, three Ci-related transcription factors [Gli1, Gli2, and Gli3] have been identified [Ruppert et al. 1990]. Glioblastoma-3 (Gli3) is processed to a repressor form (Gli3Rep) in a manner similar to Ci [Dai et al. 1999, Ruiz-I-Altaba 1999, Shin et al. 1999, Wang et al. 2000], whereas Gli1 is not [Dai et al. 1999]. Overexpression of Gli1 in cultured cells or transgenic embryos can induce transcription of Hh target genes in the absence of Hh activity [Hynes et al. 1997; Sasaki et al. 1997; Ruiz-I-Altaba 1999]. Sonic hedgehog [Shh] up-regulates Gli1 transcription but down-regulates Gli3 expression [Marigo et al. 1996; Lee et al. 1997]. Molecular analysis suggests that Gli3 can be processed into a repressor form (Gli3Rep) that suppresses the Gli1 promoter, whereas the full-length form of Gli3 [FL-Gli3] directly mediates the activation of a Gli1 promoter in response to a Shh signal [Dai et al. 1999]. Gli3 plays an important role in the development of limb bud, and mice with a mutation in Gli3 have dominant preaxial polydactyly [Hui and Joyner 1993].

Ski and its related protein Sno act as corepressors, and directly bind to two other corepressors, N-CoR/SMRT and mSin3A (Nomura et al. 1999). These three corepressors (N-CoR/SMRT, mSin3, and Sno) form a complex with histone deacetylases (HDACs) and are necessary for the transcriptional repression mediated by nuclear hormone receptors, Mad, and possibly other repressors. Ski also directly binds to Smad proteins, which induce the transcription of target genes on TGF-β [tumor growth factor] stimulation [Massagüe and Wotton 2000]. By recruiting the HDAC complex to Smad proteins, Ski inhibits TGF-β signaling. The ski-deficient mice display various abnormalities of pattern formation depending on the genetic background [Berk et al. 1997; Colmenares et al. 2002]. However, the molecular mechanism of these defects remains unknown. In this study, we have demonstrated that Ski is required for the Gli3Rep-mediated repression, and it negatively regulates the FL-Gli3-induced transcriptional activation.

Results and Discussion
Identification of Ski as a Gli3Rep-binding protein
To identify the Gli3Rep-interacting factor(s), we performed yeast two-hybrid screening using the N-terminal region of Gli3 or Gli2 as bait. Five Ski clones and three Sno clones were isolated, suggesting that Ski might play an important role in Gli3-mediated transcriptional regulation. To identify the Ski-interacting region in Gli3, we performed the glutathione S-transferase (GST) pull-down assay using various forms of in vitro translated Gli3 and GST–Ski fusion (Fig. 1A). The N-terminal region of Gli3 contains the repressor domain, whereas the C-terminal half contains the activation domain [Dai et al. 1999]. The results indicated that the repressor domain of Gli3 [amino acids 1–397] interacts with Ski. Because a deletion of one-third of the C-terminal proximal side of the repressor domain partly decreased affinity for Ski, the repressor domain may have multiple binding sites for Ski. Similar to the case of Gli3, Ski also bound to the N-terminal repressor domain of Gli2 [Fig. 1A]. To identify the Gli3-interacting domain in Ski, we used various forms of in vitro translated Ski in GST pull-down assays with a GST fusion of the repressor domain of Gli3 [Gli3CT2, Fig. 1B]. The results indicated that the region between amino acids 197 and 261 of Ski mediates the interaction with Gli3CT2. This region shows a high degree of homology (63%) with Sno. Consistent with this, Sno was also capable of binding efficiently to Gli3CT2 [data not shown].

[Keywords: Ski corepressor, Shh signaling, Gli3, Gli1 promoter, limb bud]
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Article and publication are available at http://www.genesdev.org/cgi/doi/10.1101/gad.1017302.

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GENES & DEVELOPMENT 16:2843–2848 © 2002 by Cold Spring Harbor Laboratory Press ISSN 0890-9369/02 $5.00, www.genesdev.org 2843

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**Figure 1.** Binding of Ski to Gli3 and Gli2. (A) The repressor domains of Gli3 and Gli2 bind to Ski. The relative binding activities are designated +, +, and −, which indicate the binding of 5%–9%, 3%, and <0.5% of the input protein, respectively. In the lower-left panel, the GST–Ski fusion and GST proteins that bound to the glutathione beads were analyzed by SDS-PAGE followed by Coomassie blue staining. In the lower-right panel, the in vitro translated Gli3 and Gli2 derivatives [input] and those that bound to GST–Ski were analyzed by SDS-PAGE followed by autoradiography. In the input lanes, the amount of each Gli3 derivative was 10% of that used for the binding assay. (B) Identification of the Gli3-binding domain in the Ski molecule. Binding of various forms of in vitro translated Ski to the GST–Gli3CT2 resin containing N-terminal 397 amino acids of Gli3 was examined. The relative binding activities are designated + and −, which indicate the binding of 3%–5% and <0.5% of the input protein, respectively. The band indicated by an asterisk is a GST–Gli3CT2 degradation product.

**Figure 2.** In vivo association between Gli3 and the Ski–HDAC1 complex. (A) Coimmunoprecipitation of Ski with Gli3. Lysates were prepared from 293T cells transfected with the Ski and Flag–FL-Gli3 expression plasmids together with the PKA expression plasmid [+] or −. Lysates were precipitated by anti-Ski or anti-β-galactosidase antibodies, and the immunocomplexes were analyzed by Western blotting using anti-Flag antibodies. Samples from the lysates were also used directly for Western blotting. Similar experiments were also done using the Ski and Flag–Gli1 expression plasmids [right]. (B) Two hybrid assays in mammalian cells. MNS70 cells were cotransfected with the Gli site-containing reporter, the plasmid to express FL-Gli3 or GLI3ΔC, and the Ski–VP16 expression plasmid. The average degree of activation observed in two experiments is indicated with standard deviations. (C) Coimmunoprecipitation of HDAC1 with Gli3. Lysates were prepared from 293T cells transfected with the Ski and Flag–FL-Gli3, or Flag–Gli3ΔC, and cells were then immunostained with the corresponding antibodies. Localizations were revealed by staining with rhodamine- or FITC-conjugated secondary antibodies. The signals were analyzed by deconvolution microscopy, and representative optical sections are shown. (Bottom) CV-1 cells were transfected with the Ski expression plasmid to express Flag–Ski, Flag–FL-Gli3, or Flag–Gli3ΔC, and cells were then immunostained with the corresponding antibodies. Localization was revealed by staining with rhodamine- or FITC-conjugated secondary antibodies. The signals were analyzed by deconvolution microscopy, and representative optical sections are shown.

Ski binds to both Gli3rep and FL-Gli3

To investigate the interaction between Ski and Gli3 in mammalian cells, we performed coimmunoprecipitations using 293T cells [Fig. 2A]. When FL-Gli3 was coexpressed with the catalytic subunit of cAMP-dependent protein kinase (PKA), FL-Gli3 was efficiently processed to FL-Gli3rep as reported [Dat et al. 1999]. The anti-Ski antibodies coprecipitated both FL-Gli3 and Gli3rep, whereas control anti-β-galactosidase antibody did not. In similar experiments, Gli1 was not coprecipitated with Ski. In addition, a two-hybrid assay was performed in mammalian cells using the Ski–VP16 fusion, which consists of the N-terminal 492 amino acids of Ski and the VP16 transcriptional activation domain [Fig. 2B]. The Gli site-containing luciferase reporter and the Ski–VP16 expression plasmid were transfected into MNS70 cells together with the plasmids expressing either FL-Gli3 or Gli3ΔC containing the N-terminal 649 amino acids of Gli3, which has a structure similar to Gli3rep. The Ski–VP16 fusion stimulated FL-Gli3 activity 3.9-fold [relative luciferase activity: 0.74 and 2.87] and Gli3ΔC activity by 18.8-fold [relative luciferase activity: 0.19 and 3.57; see Supplementary Table 1]. These results indicate that Ski interacts with the N-terminal region of Gli3.

To further confirm the Ski–Gli3 interaction, we investigated the colocalization of both proteins in CV-1 cells [Fig. 2C]. When Ski was expressed alone, it was localized to a dot-like nuclear structure, as reported [Nomura et al.
Role of Ski in pattern formation

Interaction of Ski with Gli3<sup>Rep</sup> suggested that Ski is required for the Gli3<sup>Rep</sup>-dependent transcriptional repression. We investigated whether the Ski mutants abrogate Gli3<sup>Rep</sup>-dependent repression in a dominant negative fashion using a neural stem cell line, MNS-70, that is able to express different sets of ventral-specific genes in response to Shh (Fig. 3A). In the luciferase reporter assays, the Gal4–Gli3CT2 fusion, which consists of the Gal4 DNA-binding domain fused to the N-terminal repressor domain of Gli3, strongly repressed transcription from the Gal4 site-containing reporter. Gal4–Gli3CT2-induced repression was abrogated by the C-terminal deleted form of Ski (Δ493–728) in a dose-dependent manner, neither by the wild-type Ski nor by the N-terminal deleted form (Δ46–260), which cannot bind to Gli3. Because the C-terminal deleted form of Ski binds to Gli3 but not to corepressor mSin3A, it may disrupt the Gli3-corepressors-HDAC complex. SkiΔ46–260 may not efficiently mask the surface of mSin3A molecule in vivo because the mSin3A forms a complex with many other proteins. We also performed luciferase reporter assays using mouse embryonic fibroblasts (MEFs) prepared from wild-type or Ski-deficient embryos (Shinagawa et al. 2001, Fig. 3B). Gal4–Gli3CT2 efficiently repressed luciferase expression from the Gal4 site-containing reporter in wild-type MEFs, but not in Ski-deficient MEFs. In addition, similar results were obtained by using Gal4–Gli2N containing the N-terminal 308 amino acids of Gli2. Thus, a loss of Ski abrogated the Gli3CT2- or Gli2N-induced transcriptional repression, suggesting that the amounts of Sno in MEFs are relatively low. In fact, we found that the relative levels of Sno compared with Ski are lower in MEFs than that in E12.5 embryos (Supplementary Fig. 1).

To further confirm that Ski is required for Gli3<sup>Rep</sup>-dependent repression, antibodies were coimmunoprecipitated from E11.5 mouse embryos with HDAC complex. Ski was cotransfected with the reporter plasmid containing the TK promoter and the Gal4-binding sites, and/or the Gal4–Gli3CT2 expression plasmid (Fig. 3C).

Injection of the reporter alone into Rat-1 cells gave rise to many lacZ-positive cells. Cointegration of this lacZ reporter with the Gal4–Gli3CT2 expression plasmid resulted in a decrease in the number of lacZ-positive cells. This decrease was relieved partially by coinjection of anti-Ski or anti-Sno antibodies, and more significantly by coinjection of both antibodies. The incomplete abrogation of Gal–Gli3CT2 function even after coinjection of both antibodies may be due to the presence of other Ski-related proteins. Coinjection of both antibodies did not affect the decrease in the number of lacZ-positive cells mediated by Gal-5EF1, which was previously shown not to use Ski/Sno (Nomura et al. 1999).
Ski negatively regulates the Shh-induced activation of Gli1 promoter mediated by FL-Gli3

Ski binds not only to Gli3Rep but also to FL-Gli3. We examined whether Shh- and FL-Gli3-dependent activation of the Gli1 promoter is inhibited by Ski [Fig. 3D]. As reported [Dai et al. 1999], coexpression of Shh and Gli3 in MNS-70 cells transfected with the Gli1 promoter luciferase reporter enhanced the luciferase expression. Coexpression of Ski inhibited this activation in a dose-dependent manner. Thus, Ski also inhibits Shh- and FL-Gli3-dependent activation of the Gli1 promoter. We further investigated whether Ski inhibits the Shh-dependent endogenous Gli1 induction mediated by Gli3 in MNS-70 cells [Fig. 3E]. As reported previously [Dai et al. 1999], ectopic expression of Shh alone or together with Gli3 in transfected MNS-70 cells induces expression of the endogenous Gli1 gene 5.2- and 10.2-fold, respectively. Coexpression of c-Ski with Shh and Gli3 significantly lowered the level of induction of Gli1 mRNA by about 3.8-fold. These results further confirm that c-Ski negatively regulates the Shh-dependent transcriptional activation of Gli1.

Genetic interaction between Ski and Gli3

To test for a genetic interaction between Ski and Gli3, we analyzed the skeletons of limbs of double mutant mice (Fig. 4A; Table 1). Genetic interaction between Ski and Gli3 was determined in the anterior region of limb buds. To examine this, we analyzed the expression of Gli1 by in situ hybridization [Fig. 4A]. In wild-type and Ski−/+ forelimb buds, Gli1 was expressed only in the posterior region, whereas in Gli3XtJ/+; Ski−/+ forelimb buds, it was also weakly expressed in the anterior region. The level of Gli1 expression in the anterior region of the Gli3XtJ/+; Ski−/+ limb bud appeared to be lower than that in Gli3XtJ/+ limb buds, but higher than that in Gli3XtJ/Gli3XtJ and Ski−/+ limb buds. To accurately measure the Gli1 expression level, we prepared RNA from the anterior one-third and posterior one-third regions of limb buds, and quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was performed [Fig. 4B]. Gli1 expression levels in the anterior region of Gli3XtJ/+; Ski−/+ and Gli3XtJ/Gli3XtJ limb buds were 115% and 125% higher than those of wild type, respectively, whereas there was no apparent difference in the Gli1 mRNA level between Gli3XtJ/+ and wild-type limb buds. Further, there was also no apparent difference in the anterior Gli1 mRNA level between Gli3XtJ/Gli3XtJ and Gli3XtJ/Gli3XtJ, Ski−/+ [data not shown]. Although Shh is ectopically expressed in the posterior one-third region of limb buds. To examine this, we analyzed the expression of Gli1 by in situ hybridization [Fig. 4A]. In wild-type and Ski−/+ forelimb buds, Gli1 was expressed only in the posterior region, whereas in Gli3XtJ/+; Ski−/+ forelimb buds, it was also weakly expressed in the anterior region. The level of Gli1 expression in the anterior region of the Gli3XtJ/+; Ski−/+ limb bud appeared to be lower than that in Gli3XtJ/+ limb buds, but higher than that in Gli3XtJ/Gli3XtJ and Ski−/+ limb buds. To accurately measure the Gli1 expression level, we prepared RNA from the anterior one-third and posterior one-third regions of limb buds, and quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was performed [Fig. 4B]. Gli1 expression levels in the anterior region of Gli3XtJ/+; Ski−/+ and Gli3XtJ/Gli3XtJ limb buds were 115% and 125% higher than those of wild type, respectively, whereas there was no apparent difference in the Gli1 mRNA level between Gli3XtJ/+ and wild-type limb buds. Further, there was also no apparent difference in the anterior Gli1 mRNA level between Gli3XtJ/Gli3XtJ and Gli3XtJ/Gli3XtJ, Ski−/+ [data not shown]. Although Shh is ectopically expressed in the

Figure 4. Genetic interaction between Ski and Gli3. (A) Skeletal phenotype of the forelimbs and expression of the Gli1 and Shh in the forelimb bud. [Left two panels] Ventral and dorsal views of the forelimbs of E17.5 fetuses and newborn mice. Extra digits are shown by asterisks. A small posterior outgrowth is indicated by an arrowhead. Anterior is up. [Right three panels] Expression patterns of Gli1 and Shh in E11.5 and E12.5 forelimb buds. Close-up view of whole-mount in situ hybridization of the right forelimbs is indicated. (B) Level of Gli1 mRNA. Total RNA was prepared from the anterior and posterior parts of the E11.5 limb buds, and Gli1 mRNA was measured by the quantitative real-time PCR. The relative amount of Gli1 mRNA compared with wild type is indicated by a bar graph. The level of Gli1 mRNA in the posterior part was 10.6-fold higher than that in the anterior part. (C) Expression of Ski and Su2o in the wild-type E11.5 and E12.5 limb buds. Close-up view of whole-mount in situ hybridization of the right forelimb buds is shown.

Ectopic expression of Gli1 mRNA correlates with extra digits in Gli3XtJ/+; Ski−/+ mice

Because Shh blocks the Gli3 processing, the levels of Gli3Rep protein are higher in the anterior limb buds than in the posterior limb buds [Wang et al. 2000]. Therefore, one possibility for the enhanced digit abnormalities of Gli3XtJ/+; Ski−/+ mice is that Gli3Rep represses a subset of target genes by interacting with Ski in the anterior region of limb buds. To examine this, we analyzed the expression of Gli1 by in situ hybridization [Fig. 4A]. In wild-type and Ski−/+ forelimb buds, Gli1 was expressed only in the posterior region, whereas in Gli3XtJ/+; Ski−/+ forelimb buds, it was also weakly expressed in the anterior region. The level of Gli1 expression in the anterior region of the Gli3XtJ/+; Ski−/+ limb bud appeared to be lower than that in Gli3XtJ/+ limb buds, but higher than that in Gli3XtJ/Gli3XtJ and Gli3XtJ/Ski−/+ limb buds. To accurately measure the Gli1 expression level, we prepared RNA from the anterior one-third and posterior one-third regions of limb buds, and quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was performed [Fig. 4B]. Gli1 expression levels in the anterior region of Gli3XtJ/+; Ski−/+ and Gli3XtJ/Gli3XtJ limb buds were 115% and 125% higher than those of wild type, respectively, whereas there was no apparent difference in the Gli1 mRNA level between Gli3XtJ/+ and wild-type limb buds. Further, there was also no apparent difference in the anterior Gli1 mRNA level between Gli3XtJ/Gli3XtJ and Gli3XtJ/Gli3XtJ, Ski−/+ [data not shown]. Although Shh is ectopically expressed in the

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CREB-binding protein; Akimaru et al. 1997; Dai et al. 1999). The N-terminal 613 amino acids of GLI3 or the N-terminal 641 amino acids of Gli2 were used as bait.

In vitro binding assays and coimmunoprecipitation
In vitro binding assays were done using GST–Ski and GST–Gli3CT2 as described [Dai et al. 1999]. For coimmunoprecipitation, a mixture of the plasmids to express Gli3, Gli1, Ski, HDAC1, or PKA was transfected into 293T cells. Forty hours after transfection, cells were lysed, and lysates were immunoprecipitated using appropriate antibodies. The immunocomplex was analyzed by Western blotting using appropriate antibodies. For coimmunoprecipitation of endogenous proteins, the lysates were prepared from the 11.5-dpc mouse fetuses and immunoprecipitated using anti-Ski antibody, followed by Western blotting using anti-GLI3.

Mammalian two-hybrid assays, subcellular localization, and antibody injection assays
The mammalian two-hybrid assays were done using the Gli-binding sites containing luciferase reporter [Sasaki et al. 1997] and the plasmid encoding a Ski–Vp16 fusion. The subcellular localization study and antibody injection assays were done essentially as described [Nomura et al. 1999].

Luciferase reporter assays and analysis of Gli1 expression
The luciferase reporter assays using the luciferase reporter containing the Gal4 site or the Gli1 promoter [pHR-luc] were done as described [Dai et al. 1999]. Gli1 gene expression in MNS-70 cells were also examined as described [Dai et al. 1999].

Analysis of embryos and quantitative real-time PCR
Analysis of cartilaginous tissues of newborn mice and whole-mount in situ hybridization was performed essentially as described [Tanaka et al. 1997]. Quantitative real-time PCR-based measurements of RNA abundance were carried out using gene-specific double fluorescent probes and LightCycler [Roche].

Acknowledgments
We thank J. Aruga for Gli3–Gfp–mice, B. Vogelstein for the Gli cDNAs, S.L. Schreiber for the HDAC1 cDNA, M. Nakafuku for MNS-70 cells, S. Noji for the Shh cDNA, and members of Experimental Animal Division of RIKEN Tsukuba Institute for maintenance of the mice. This work was supported, in part, by the grants from the Ministry of Education, Science and Technology [S.1], Human Frontier Science Program [S.1], and National Institutes of Health (C.C.).

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*Genes Dev.* 2002, 16:
Access the most recent version at doi:10.1101/gad.1017302

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