Sonic Hedgehog-induced Activation of the Gli1 Promoter Is Mediated by GLI3*

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Ping Dai‡, Hiroshi Akimaru‡§, Yasunori Tanaka‡, Toshio Maekawa‡§, Masato Nakafuku‡§, and Shunsuke Ishii‡§

From the §Laboratory of Molecular Genetics, Tsukuba Life Science Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan, the ¶Division of Neurobiology, Department of Neuroscience, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and §CREST, Japan Science and Technology

Drosophila transcription factor cubitus interruptus (Ci) and its co-activator CRE (cAMP response element)-binding protein (CBP) activate a group of target genes on the anterior-posterior border in response to hedgehog protein (Hh) signaling. In the anterior region, in contrast, the carboxyl-truncated form of Ci generated by protein processing represses Hh expression. In vertebrates, three Ci-related transcription factors (glioblastoma gene products (GLIs) 1, 2, and 3) were identified, but their functional difference in Hh signal transduction is unknown. Here, we report distinct roles for GLI1 and GLI3 in Sonic hedgehog (Shh) signaling. GLI3 containing both repression and activation domains acts both as an activator and a repressor, as does Ci, whereas GLI1 contains only the activation domain. Consistent with this, GLI3, but not GLI1, is processed to generate the repressor form. Transcriptional co-activator CBP binds to GLI3, but not to GLI1. The trans-activating capacity of GLI3 is positively and negatively regulated by Shh and CBP-dependent protein kinase, respectively, through a specific region of GLI3, which contains the CBP-binding domain and the phosphorylation sites of CBP-dependent protein kinase. GLI3 directly binds to the Gli1 promoter and induces Gli1 transcription in response to Shh. Thus, GLI3 may act as a mediator of Shh signaling in the activation of the target gene Gli1.

Hedgehog protein (Hh)1 plays an important role in the pattern formation of many species including insects and vertebrates (for review, see Refs. 1 and 2). The hh gene was originally identified as a segment polarity gene in Drosophila. Hh is a secreted and diffusible protein and is a critical signaling molecule for the pattern formation of the anterior-posterior (A-P) axis. Hh is expressed only in the posterior compartment of Drosophila, which has a homology with the vertebrate family of glioblastoma (Gli) transcription factors (9), mediates Hh-induced transcriptional activation (10). On the other hand, in the anterior region, located far from the A-P border, Ci is processed into a C-terminal truncated form that functions as a repressor of hh expression (11, 12). Hh signaling suppresses this processing on the A-P border. Also on the A-P border, extracellular Hh binds to the transmembrane protein Patched (Ptc), preventing its normal inhibition of Smoothened (Smo), another transmembrane protein of the receptor complex (13–16). This allows Smo to transduce a signal to Ci through the positive regulator Fused (Fu), which is a serine-threonine kinase (17). Hh signaling also negates the inhibitory effects of Costal2 (Cos2), a kinesin-related protein (18, 19), cAMP-dependent protein kinase (PKA) (20–24), and Suppressor of fused (Su(fu)) (25). Ci, Fu, Su(fu), and Cos2 are normally found in a complex that docks at microtubules, and tight binding of this complex to microtubules is prevented by Hh signaling (18, 19, 25) These facts suggest that Hh signaling prevents association of the complex with the cytoskeleton and cleavage of Ci into its repressor form.

The most studied member of the hh multigene family in vertebrates is Sonic hedgehog (Shh). Targeted disruption of mouse Shh leads to multiple defects in embryonic tissues, including the notochord, floor plate, and limb structures (26). The mechanism of Shh signaling is thought to resemble to that of Drosophila hh. For instance, the negative regulation of Shh signaling by PKA was also observed in vertebrate embryos (27). It was further demonstrated that suppression of PKA activity is sufficient to activate targets of the Shh signaling pathway in the vertebrate central nervous system (28). The three vertebrate genes Gli1, Gli2, and Gli3 have a homology with ci (29, 30). All three GLI proteins bind to the consensus sequence 5′-TGGGTGGTC-3′ through their metal finger regions (31, 32). Gli1 is downstream of Shh, and ectopic Shh signaling induces its expression, whereas expression of Gli3 is down-regulated by ectopic Shh signaling (33–35). In fact, Gli1 and Ptc, both of which are downstream of Shh, are expressed in similar domains in diverse regions of developing mouse embryo (36), and ectopic expression of Gli1 in Xenopus and mouse embryos induced the expression of midline neural plate markers, such as Hnf-3β (34, 35), indicating that Gli1 is a positive regulator of Shh signaling. As in the case of Ci, which acts as a repressor in the anterior compartment, GLI3 also represses Shh expression in the anterior region of the limb bud (37). Moreover, GLI1 is an activator of transcription, whereas GLI3 represses transcription of an Hnf-3β enhancer element (38, 39). Although these facts suggest functional differences between GLI1 and GLI3 proteins, their precise roles in Shh signaling remain unclear.

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‡ To whom correspondence should be addressed. Tel.: 81-298-36-9031; Fax: 81-298-36-9030; E-mail: sishi@rtc.riken.go.jp.

1 The abbreviations used are: CBP, CRE (cAMP response element)-binding protein; Ci, cubitus interruptus gene product; GLI, glioblastoma gene product; Hh, hedgehog protein; PKA, cAMP-dependent protein kinase; Shh, Sonic hedgehog gene product; A-P, anterior-posterior; Ptc, Patched transmembrane receptor; Hnf-3β, hepatocyte nuclear factor-3β; Shh, Sonic hedgehog; Cre, basic helix-loop-helix; CMV, cytomegalovirus; Shh, Sonic hedgehog; Hh, hedgehog protein.

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Translocations and mutations of the human GLI3 gene cause abnormal pattern formation through haploinsufficiency in humans and mice. The associated human syndromes are known as Greig cephalopolysyndactyly syndrome (GCPs) (40) and Pallister-Hall syndrome (PHS) (41), and the mouse mutants such as extra-toe (Xt) have the mutations in the Gli3 gene (42). Mutations or deletions of the human Cbp gene at chromosome 16p13.3 also cause the Rubinstein-Taybi syndrome (RTS) through haploinsufficiency (43). RTS consists of a wide variety of developmental defects, including craniofacial malformations, broad thumbs, broad big toes, and mental retardation (44). Disruption of one copy of the mouse Cbp gene by gene targeting causes skeletal abnormality partially resembling that of RTS (45). CBP was originally identified as a transcriptional co-activator of CREB (46, 47) and has recently been found to be required for many other transcription factors (for review, see Ref. 48). RTS and GCPs are distinct syndromes, but both include preaxial limb anomalies and craniofacial features. A recent genetic study of Drosophila CBP mutants indicated that CBP is used as a co-activator of Ci and plays an important role in hh signaling (49). The structural homology of Ci and GLI3 supports the idea that CBP acts as a co-activator of GLI3 in vertebrates. As described above, however, GLI3 was reported to be a repressor of Shh transcription, not an activator of Shh targets.

To investigate the role of CBP in vertebrate pattern formation, we have examined whether CBP directly interacts with human GLI3 and GLI1. Our results indicate that GLI3 and GLI1 have multiple differences in their domain structures including the CBP-binding domain. CBP binds only to GLI3. Furthermore, a specific region in the GLI3 protein that includes the CBP-binding domain can mediate Shh-induced trans-activation. We have also demonstrated that GLI3 directly binds to the mouse Gli1 promoter and mediates Shh-induced activation of the Gli1 promoter in Shh-responsive multipotential neural stem (MNS)-70 cells. Thus, our results demonstrate distinct roles for GLI3 and GLI1 in Shh signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The human GLI1 and GLI3 cDNA was kindly provided by Drs. Kenneth W. Kinzler and Bert Vogelstein. The plasmids used for in vitro transcription/translation of the various forms of human GLI3 were generated by inserting various fragments of GLI3 cDNA, which were prepared by the polymerase chain reaction (PCR)-based method, into the Neo-I-Xbal site of pSPUTK (Stratagene). For all constructs generated by PCR, it was confirmed by sequencing that they do not contain mutations. The pA10CAT6GBS reporter plasmid containing the GLI3-binding sites was constructed by inserting six tandem copies of the GLI3-binding site (5'-GCGTGGACCAACGAAATT-3') (32) into the BglII site of pA10CAT2, in which the SV40 early promoter was linked to the CAT gene (50). The plasmids pSR-GLI3 and pSR-GLI1 to express human GLI3 and GLI1 in cultured cells were made by placing the GLI3 and GLI1 cDNA downstream of the SRE promoter, respectively (51). To generate the plasmids pACT-GLI3 and pACT-GLI1 encoding the Flag-linked GLI3 and GLI1, the synthetic oligonucleotides containing one copy of the Flag sequence were inserted downstream of the chicken cytoplasmic β-actin promoter and fused in frame to the N terminus of GLI3 and GLI1, respectively. The plasmids to express Gal4-GLI3 and Gal4-GLI1, in which various portions of GLI3 and GLI1 were fused to the DNA-binding domain of Gal4 (amino acids 1–147), were constructed by the PCR-based method using the CMV promoter-containing vector. The two Gal4-GLI3 fusions containing the N-terminal region or the CBP-binding domain contained amino acids 1–397 and 827–1132 of GLI3, respectively. The luciferase reporter plasmid containing the mouse Gli1 promoter was generated by inserting the 3.5-kilobase HindIII-EcoRI fragment containing the Gli1 promoter upstream of the luciferase gene in the plasmid pGL3-basic vector (Promega). The Gli1 promoter fragment was kindly provided by Drs. Heidi Park and Alexandra L. Joyner. The mutant Gli1 promoter, in which the eight GLI3-binding sites were disrupted, was constructed by the PCR-based method. The plasmids to express CBP and E1A were described previously (52). The Shh expression plasmid pJT4/Shh and the Gal4 site-containing luciferase reporter, in which three tandem repeats of Gal4 sites were linked to the thymidine kinase promoter, was kindly provided by Drs. Sumihare Noji, Kazuhiko Umesono, and Ronald M. Evans, respectively.

**GST Pull-down Assay and Co-immunoprecipitation**—The GST pull-down assay using the GST-CBP and the in vitro translated GLI3 was performed as described (52).

For co-immunoprecipitation, a mixture of 5 μg of the CBP expression plasmid pCBP-Flag and 5 μg of the Flag-linked GLI3 expression plasmid pact-Flag-GLI3 or the Flag-linked GLI1 expression plasmid pact-Flag-GLI1 were transfected into 293T cells. Forty hours after transfection, cells were lysed in the lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 10 mM NaF, 0.5% Nonidet P-40), and whole cell lysates were prepared. Lysates were immunoprecipitated using anti-CBP antibodies CT (Upstate Biotechnology) and A-22 (Santa Cruz), and the immune complexes were separated on 7% SDS-polyacrylamide gels and analyzed by Western blotting using anti-Flag antibodies (Upstate Biotechnology) and ECL detection reagents (Amersham Pharmacia Biotech).

**Transient Co-transfection Assays**—The amount of each plasmid DNA used for transfection is described in the legends to the figures. The transfection experiments and CAT assays were performed as described (52). Dual luciferase assays were done as described by the supplier.

**Examination of Proteolysis of Gli1 and Gli3 Proteins**—Extracts were prepared from mouse embryos at 10.5 days post-coitus as described (45), and separated on 7 and 10% gels to detect GLI3 and GLI1, respectively. Western blotting was performed using anti-GLI3 and anti-GLI1 antibodies (Santa Cruz). The anti-GLI3 antibody that recognizes the N-terminal region of GLI3 was further purified by using the GST-GLI3 affinity resin.

To examine the processing of GLI3 in cultured cells, 4 μg of the N-terminal Flag-linked GLI3 expression plasmid, pact-Flag-GLI3, or the GLI1 expression plasmid, pSR-Shh, was transfected into 293T cells without 1 μg of pCMV-β-gal plasmid expressing a CMV-promoter containing the β-galactosidase expression plasmid to express the catalytic subunit of PKA. Forty hours after transfection, cells were lysed in the lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 10 mM NaF, 0.5% Nonidet P-40), and whole cell lysates were prepared. Lysates were separated on 7% SDS-polyacrylamide gels, and analyzed by Western blotting using anti-Flag antibodies (Upstate Biotechnology), anti-GLI1 antibodies (Santa Cruz), and ECL detection reagents (Amersham Pharmacia Biotech).

**Induction of Endogenous Gli1 Gene Expression by Shh in MNS-70 Cells**—MNS-70 cells were transfected with a mixture of 4 μg of the Shh expression plasmid pJT4/Shh and 4 μg of the plasmid to express the GLI3-binding domain of CBP (amino acids 454–718) or GLI3, or the control plasmid lacking the cDNA to be expressed. Forty hours after transfection, total RNA was prepared using TRIZOL (Life Technologies, Inc.), and reverse transcription-PCR was performed as described by Sasaki et al. (38) to measure Gli1 mRNA and cytoplasmic β-actin mRNA as a control.

**RESULTS**

**Binding of CBP to GLI3 but not to GLI1**—We first examined whether human GLI3 and GLI1 could interact directly with mouse CBP in vitro (Fig. 1A). The full-length form of human GLI3 was in vitro translated and mixed with the GST-CBP resin containing the 265-amino acids region of CBP, which is responsible for binding to multiple transcription factors, including phospho-CREB, e-Jun, and e-Myc (46, 52, 53). GLI3 efficiently bound to GST-CBP but not to the GST resin alone (Fig. 1A, left panel). PKA treatment of GLI3 did not increase the efficiency of binding of GLI3 to CBP. To examine the possibility that the in vitro translated GLI3 is already phosphorylated by some kinase(s), we treated the in vitro translated GLI3 with [γ-32P]ATP and phosphorylated protein. The phosphorylation treatment did not affect the binding of GLI3 to CBP. In contrast to GLI3, in vitro translated GLI1 protein did not bind to CBP in the GST pull-down assay using the same GST-CBP resin (Fig. 1A, right panel). GLI1 also did not bind to the GST-CBP fusions containing various other regions of CBP, which covered the entire CBP molecule (data not shown). These results indicate

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2 H. Park and A. L. Joyner, manuscript in preparation.
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A possible in vivo interaction between GLI3 and CBP was investigated by co-immunoprecipitation (Fig. 1B). Whole cell lysates were prepared from 293T cells transfected with the two plasmids to express CBP and Flag-linked GLI3, and incubated with anti-CBP antibodies or a control antibody, anti-β-galactosidase. The immunoprecipitates were separated on an SDS-polyacrylamide gel and the N-terminal Flag-linked GLI3 was detected by Western blotting using the anti-Flag antibody. A significant amount of Flag-GLI3 was co-precipitated with the CBP C-terminal region-specific antibody (CT), whereas the CBP N-terminal region-specific antibody (A-22) co-precipitated only a small amount of Flag-GLI3. The low efficiency of co-immunoprecipitation with the A-22 antibody could be due to the proximity of the epitope for this antibody with the GLI3-binding domain in the CBP molecule. The control antibody, anti-β-galactosidase, did not co-immunoprecipitate Flag-GLI3.

To investigate which domain of GLI3 interacts with CBP, we used a series of deletion mutants of GLI3 in the in vitro binding assay (Fig. 1C). Various forms of GLI3 were synthesized using an in vitro translation system, mixed with the GST-CBP affinity resin, and the bound proteins were analyzed. The results showed that the 306-amino acid region between amino acids 827 and 1132 in the C-terminal half of GLI3 bound to CBP with almost the same efficiency as full-length GLI3.

By using a series of GST-CBP proteins containing various portions of the CBP molecule, we confirmed that regions other than the N-terminal region containing the CREB-binding domain (KIX: the region in CBP molecule that binds to the kinase-inducible activation domain (KID) of CREB) did not bind to GLI3 (data not shown). This region of CBP is known to directly interact with multiple transcription factors, such as CREB and c-Myb (52). To map the GLI3-binding domain more precisely, we used three additional GST-CBP resins for the GST-pull down assay (Fig. 1D). This revealed that the region

![Diagram](image)

**Fig. 1. Binding of CBP to GLI3 but not to GLI1.** A, GST pull-down assay. At the top, GLI3 and GLI1, which share 88% identical amino acids in their metal finger regions, are schematically shown. The GST-CBP fusion containing the N-proximal region of CBP (amino acids 454-718) or the GST resin alone was mixed with the in vitro translated ([35S]GLI3 or [35S]GLI1, and the bound proteins were analyzed by 10% SDS-PAGE. To examine the effect of PKA, [35S]GLI3 or [35S]GLI1 was treated with PKA (+PKA) or control buffer (−PKA) and used for the binding assay. To investigate the effect of phosphatase, the binding of GLI3 to CBP was similarly analyzed with (+APase) or without (−APase) treatment of [35S]GLI3 by potato acid phosphatase. The amount of GLI3 protein bound to GST-CBP was about 18% of the input protein, whereas the GST control resin bound to less than 1% of the input protein (data not shown). B, co-immunoprecipitation. Whole cell lysates were prepared from 293T cells transfected with the two plasmids to express CBP or the Flag-linked GLI3, and used for immunoprecipitation with the anti-CBP antibody CT, A-22, or the control antibody anti-β-galactosidase. The immunocomplexes were analyzed by Western blotting using the anti-Flag antibody. In the left lane (Western), an aliquot of whole cell lysate was directly used for Western blotting. C, identification of the CBP-binding domain in GLI3. The structures of the various forms of GLI3 used are shown below. The GST pull-down assays were performed as described above, and the results of binding assays shown below are indicated on the right. The binding activities of the mutants are designated + and −, which indicate the binding of 15–20% and 1% of the input proteins, respectively. In the input lanes, various forms of GLI3 indicated above each lane were synthesized in vitro and analyzed by 10% SDS-PAGE. Various forms of GLI3 synthesized were mixed with the GST-CBP resin, and the bound proteins are shown on the right half (Bound to CBP) of the bottom panel. The amount of protein in the input lanes was 10% of that used for the binding assay. The lanes for the 827/1132 protein were exposed for a longer time than the other lanes, because the efficiency of in vitro translation of this protein was relatively low. D, identification of the GLI3-binding domain in CBP. The domains of CBP are indicated at the top: C/H, cysteine- and histidine-rich domain; KIX, CBP-binding domain; Bromo, bromo domain. The structures of the various forms of CBP that were reported previously (52) are shown below. The GST pull-down assays were performed as described above, and the results of the binding assays shown below are indicated on the right. The binding activities of the mutants are designated + and −, which indicate the binding of 15–20% and 1% of the input proteins, respectively. The results of binding assays using CREB and c-Myb to each GST-CBP resin, which were previously reported (52), are also indicated.
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FIG. 2. Presence of the activation and repression domains in GLI3. A, transcriptional activation by GLI3. trans-Activation by GLI3 was examined by co-transfection assays in NIH3T3 cells using the CAT reporter containing the GLI3-binding sites. A mixture of 0, 3, 6, 9, 12, or 15 μg of the GLI3 expression plasmid pSRa-CBP, 6 μg of the CAT reporter plasmid pA10CAT6GBS, and 1 μg of the internal control plasmid pcDNA3.1(+)-galactosidase was transfected into NIH3T3 cells. The total amount of plasmid DNA was adjusted to 22 μg by adding the control plasmid DNA. The experiments were repeated three times, and the differences between the experiments were no more than 20%. Typical results are indicated by a bar graph. The shaded bar shows the data obtained in the absence of the GLI3 expression plasmid. B, potentiation of GLI3-induced trans-activation by CBP. The effect of CBP on GLI3-induced trans-activation was investigated by further addition of increasing amounts of the CBP expression plasmid. A mixture of 0, 4, 8, 12, or 16 μg of the CBP expression plasmid pSRa-CBP, 5 μg of the CAT-reporter plasmid pA10CAT6GBS, 0 or 6 μg of the GLI3 expression plasmid pSRa-GLI3, and 1 μg of the internal control plasmid pcDNA3.1(+)-galactosidase was transfected into NIH3T3 cells, and CAT assays were done. The total amount of plasmid DNA was adjusted to 27 μg by adding the control plasmid DNA. The experiments were repeated three times, and the average is shown as the relative CAT activity, with S.D. as indicated. C, repression of GLI3-dependent transcriptional activation by E1A. A mixture of 6 μg of the CAT-reporter plasmid pA10CAT6GBS, 9 μg of the GLI3 expression plasmid pSRa-GLI3, 1 μg of the plasmid to express wild type 12SE1A, ΔΔ21–150, or Δ30–85 mutant, and 1 μg of the internal control plasmid pcDNA3.1(+)-galactosidase was transfected into NIH3T3 cells, and CAT assays were done. The total amount of plasmid DNA was adjusted to 27 μg by adding the control plasmid DNA. The experiments were repeated three times, and the average is shown with S.E. The data indicating the positive regulation by Shh is shown by a shaded bar. The results of co-transfection experiments using various GLI3 deletion mutants with the GLI site-containing reporter suggested that the N-terminal region of GLI3 contains the transcriptional repression domain, whereas the CBP-binding domain mediates transcriptional activation (data not shown). To confirm this, we made the two Gal4-GLI3 fusions, which consisted of the Gal4 DNA-binding domain and the N-terminal region or CBP-binding domain of GLI3, and examined their capacity to modulate luciferase expression from the Gal4 site-containing luciferase reporter (Fig. 2D). The Gal4 fusion containing the N-terminal 397 amino acids (Gal4-N) repressed luciferase expression to 35% of the level of the control Gal4 DNA-binding domain alone (Fig. 2D, cf. lanes 1 and 2), whereas the Gal4 fusions containing the CBP-binding domain (Gal4-CBD) stimulated luciferase expression 5-fold. The expression levels of these two fusion proteins were similar (data not shown). Co-expression of CBP enhanced trans-activation by the CBP-binding domain (Fig. 2D, cf. lanes 5 and 6) but did not affect the function of the N-terminal repression domain (cf. lanes 2 and 3). E1A inhibited CBP-dependent trans-activation (cf. lanes 5 and 7). These results indicated that GLI3 has both DNA-binding domain alone as a control, 3 μg of the CBP expression plasmid pSRa-CBP, 1 μg of the E1A expression plasmid, and 1 μg of the internal control plasmid pRL-CMV, in which the sea pansy luciferase gene is linked to the CMV promoter, was transfected into NIH3T3 cells, and luciferase assays were done. The total amount of plasmid DNA was adjusted to 10 μg by adding the control plasmid DNA. The shaded bar shows the data obtained with the Gal4-GLI3 expression plasmid. E, Shh-responsiveness of GLI3. A mixture of 0, 1, 2, 3, or 4 μg of the Shh expression plasmid pJT4/Shh, 4 μg of the Gal4 site-containing reporter plasmid pGal4-TK-luc, 0.2 μg of the plasmid to express Gal4-GLI3 fusion, which contains the full-length GLI3 or the CBP-binding domain, or Gal4 DNA-binding domain alone as a control, 3 μg of the PKA catalytic subunit expression plasmid pSRa-PKA, and 1 μg of the internal control plasmid pRL-CMV, was transfected into MNS-70 cells, and the luciferase assay was performed. The total amount of plasmid DNA was adjusted to 12.2 μg by adding the control plasmid DNA. The experiments were repeated three times, and the average is shown with S.E. The data indicating the positive regulation by Shh is shown by a shaded bar.
the repression and activation domains.

GLI3 Mediates the Shh-induced Transcriptional Activation—The identification of the CBP-binding domain of GLI3 allowed us to examine the effect of Shh and PKA on the capacity of this domain to regulate transcription. For this purpose, we used a neural stem cell line, MNS-70, that is able to express different sets of ventral-specific genes including Is1-1, Nkx-2.1, and Nkx-2.2 in response to Shh (54). The Gal4 site-containing reporter and the plasmid to express the Gal4 fusion protein containing GLI3 were co-transfected into MNS-70 cells with increasing amounts of the Shh expression plasmid in the presence or absence of the PKA catalytic subunit expression plasmid (Fig. 2E). We first examined the effect of Shh and PKA on trans-activation by the Gal4 fusion containing full-length GLI3 (Fig. 2E, left panel). The Gal4-fusion containing full-length GLI3 repressed luciferase expression to 65% of the level of the control Gal4 DNA-binding domain. Co-expression of Shh increased luciferase expression in a dose-dependent manner, whereas PKA suppressed trans-activating capacity. We then performed a similar experiment using a Gal4 fusion with the CBP-binding domain (Fig. 2E, right panel). In the absence of PKA, Shh only slightly enhanced trans-activation by the CBP-binding domain. PKA negatively regulated this trans-activation, and co-expression of Shh restored this trans-activation in a dose-dependent manner in the presence of PKA. Thus, the CBP-binding domain mediates the antagonistic actions of PKA and Shh. The Shh responsiveness of full-length GLI3 in the absence of PKA may suggest that Shh may enhance GLI3 activity through not only the CBP-binding domain but also other region. On the other hand, the trans-repression mediated by Gal4 fusion containing the N-terminal repression domain was affected neither by Shh nor by PKA (data not shown).

Negative Regulation of GLI1 Activity by Shh—To compare the functional domains of GLI3 and GLI1, we next examined the functional domains of GLI1. In the CAT co-transfection experiments using the GLI site-containing reporter and NIH3T3 cells, GLI1 increased the level of CAT activity (Fig. 3A). Consistent with the results of the GST pull-down assays, co-expression of CBP did not potentiate GLI1-induced trans-activation (Fig. 3B). To identify the transcriptional activation domain in GLI1, three different portions of GLI1 were fused to the Gal4 DNA-binding domain, and their capacity to regulate transcription was investigated by co-transfection assays using the Gal4 site-containing reporter in MNS-70 cells (Fig. 3C). The expression levels of these three fusion proteins were similar (data not shown). Full-length GLI1 fusion and the Gal4 fusion containing the C-terminal 394 amino acids enhanced luciferase expression (Fig. 3C, left panel). These results indicated that GLI1 has the activation domain in its C-terminal region and does not have a repression domain. The Shh and PKA responsiveness of the Gal4 fusion containing full-length GLI1 was investigated (Fig. 3C, right panel). The full-length GLI1 activity was suppressed by Shh, whereas PKA did not affect GLI1 activity.

Processing of GLI3 but not GLI1—Our results indicated that GLI3 has both activation and repression domains, as does the Drosophila homolog Ci, whereas GLI1 has only the activation domain. Because Ci is processed into a repressor form lacking the activation domain in the anterior compartment (11), we examined GLI3 for similar processing (Fig. 4). Extracts were prepared at 11.5 days post-coitus from mouse embryos, which express both GLI3 and GLI1, and were used for Western blotting (Fig. 4A). An antibody that can recognize the C-terminal region (amino acids 1577–1596) of mouse GLI3 detected 190- and 110-kDa proteins. Analysis of the short, 110-kDa form of GLI3 by SDS-PAGE using two in vitro translated N-truncated

Fig. 3. Domain structure and Shh responsiveness of GLI1. A, transcriptional activation by GLI1. trans-Activation by GLI1 was examined by co-transfection assays in NIH3T3 cells using the CAT reporter containing the Gal4-binding sites. A mixture of 0, 4, or 8 μg of the GLI1 expression plasmid pSRα-GLI1, 4 μg of the CAT-reporter plasmid pA10CAT6GBS, and 1 μg of the internal control plasmid pACT3 were transfected into NIH3T3 cells, and CAT assays were performed. The total amount of plasmid DNA was adjusted to 13 μg by adding the control plasmid DNA pSRα. The experiments were repeated three times, and the differences between the experiments were no more than 20%. Typical results are indicated by a bar graph. The shaded bars show the data obtained in the presence of the GLI1 expression vector. B, there was no effect of CBP on GLI1-induced trans-activation. The effect of CBP on GLI1-induced trans-activation was investigated by the addition of the increasing amounts of the CBP expression plasmid. A mixture of 0, 4, or 8 μg of the CBP expression plasmid pSRα-CBP, 5 μg of the CAT-reporter plasmid pA10CAT6GBS, 0 or 5 μg of the GLI1 expression plasmid pSRα-GLI1, and 1 μg of the internal control plasmid pACT3 were transfected into NIH3T3 cells, and CAT assays were done. The total amount of plasmid DNA was adjusted to 21 μg by adding the control plasmid DNA. The experiments were repeated three times, and the average is shown as the relative CAT activity, with S.D. indicated. C, regulation of GLI1 activity by Shh and PKA. The structure of Gal4-GLI1 fusions used is indicated, and the trans-activating capacity of each construct is shown to the right. Left panel, identification of the activation domain. A mixture of 2 μg of the Gal1 site-containing reporter plasmid pGal4-TK-luc, 0.3 μg of the plasmid to express various forms of Gal4-GLI1 fusion or Gal4 DNA-binding domain alone, and 0.5 μg of the internal control plasmid pRL-CMV was transfected into NIH3T3 cells, and luciferase assays were done. The total amount of plasmid DNA was adjusted to 5 μg by adding the control plasmid DNA. Right panel, inhibition of full-length GLI1 activity by Shh. A mixture of 0, 0.5, 1, or 2 μg of the Shh expression plasmid pFT4/Shh, 2 μg of the Gal4 site-containing reporter plasmid pGal4-TK-luc, 0.5 μg of the plasmid to express Gal4-full-length GLI1 fusion or Gal4 DNA-binding domain alone as a control, 1 μg of the PKA catalytic subunit expression plasmid pSRα-PKA, and 0.5 μg of the internal control plasmid pRL-CMV was transfected into NIH3T3 cells, and the luciferase assay was performed. The total amount of plasmid DNA was adjusted to 6 μg by adding the control plasmid DNA.
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- Processing of GLI3 but not GLI1. Analysis of GLI3 in mouse embryo extracts prepared from the 11.5-day post-coitus mouse embryo. Western blotting was performed using the anti-GLI1, which recognizes amino acids 2–17 of GLI1, or anti-GLI3, which recognizes amino acids 2–19 of GLI3.

- GLI3-specific antibody recognizing the N-terminal region (amino acids 2–20) of GLI3 detected a 100-kDa protein in addition to the full-length 190-, 60-, and 50-kDa proteins detected in mouse embryonic lysates.

- When the Shh is co-expressed with Flag-GLI3 and PKA, this processing was inhibited. In the similar experiment with the GLI3 expression plasmid and the PKA expression plasmid, only the full-length form of GLI1 was detected, and the processing of GLI1 was not observed. Thus, consistent with their domain structures, GLI3, but not GLI1, is processed to generate a repressor form.

Shh-induced Activation of the Gli1 Promoter Is Mediated by GLI3—It was recently reported that the transcription of the Gli1 gene, but not the Gli3 gene, is induced by ectopic Shh signaling (15, 34, 35). These reports and our finding that GLI3, but not GLI1, has an Shh-induced activation domain suggested that GLI3 could mediate Shh-induced activation of the Gli1 promoter. To investigate this possibility, we examined whether GLI3 directly binds to the mouse Gli1 promoter region by gel mobility shift assays using the GST-GLI3 fusion protein containing the metal finger region of GLI3 (Fig. 5A). Among six DNA fragments covering the mouse Gli1 promoter region (55), only two DNA fragments, the BamHI-Xhol 210-base pair fragment (fragment A) and the adjacent XhoI-EcoRI 800-base pair fragment (fragment B) containing the two exons that encode the 5′-untranslated region of Gli1 mRNA, bound to the GST-GLI3 recombinant protein and generated the specifically retarded bands (Fig. 5A, left panel). Increasing the amount of GST-GLI3 protein generated multiple retarded bands (Fig. 5A, middle and right panels), suggesting the presence of multiple GLI3-binding sites in these two fragments. In fact, the DNA sequences of both fragments indicated the presence of three and five putative GLI-binding sites in fragments A and B, respectively (Fig. 5B, left panel). A comparison of these DNA sequences with the previously reported consensus sequence (5′-TGGGTGGTC) for the GLI-binding site (32) indicated that these eight DNA sequences have fewer than three mismatches with the reported consensus sequence. To confirm that these sites are really responsible for GLI3 binding, the sixth G residue of all of these sites was mutated to an A residue, and the mutated fragments were used for the gel retardation assays. The introduction of this mutation into these putative GLI3-binding sites abolished GST-GLI3 binding (Fig. 5B, right panel).

When either of fragment A or fragment B was inserted upstream of the luciferase gene, the resulting constructs expressed a significant level of luciferase in transfected NIH3T3 and MNS-70 cells, indicating that both of these fragments have promoter activity (data not shown). We also constructed a
to Shh. The structure of the genomic clone containing the two exons corresponding to the 5'-untranslated region of the mouse Gli1 gene is indicated at the top. B, BamHI; EcoRI; H, HindIII; K, KpnI; X, XhoI. The direction of transcription is from left to right. The eight putative GLI3-binding sites are indicated by vertical arrows. The gel mobility shift assays were performed using the GST fusion protein containing the metal finger region of GLI3 and the six DNA fragments prepared from the indicated genomic clone as probes. Of six fragments, only two, the 210-base pair BamHI-XhoI fragment (fragment A) and the 90-base pair XhoI-EcoRI fragment (fragment B), bound to GST-GLI3. The results using these two fragments are shown below. In the left panel, the 32P-labeled fragment A or fragment B was incubated with the GST-GLI3 fusion proteins (0, 40, 80, 160, or 320 ng) were incubated with the fragment A or fragment B probe. B, multiple GLI3-binding sites in the Gli1 promoter region. The DNA sequence of eight putative GLI3-binding sites in fragments A and B are indicated on the left. The previously reported consensus sequence for the GLI-binding site is shown below. On the right, the gel mobility shift assays were performed using 25 ng of GST-GLI3 protein with the wild type and mutant fragments, in which the sixth G residue in the eight putative GLI3-binding sites was changed to A. C, transcriptional activation of the Gli1 promoter by Gli3 in NIH3T3 cells. A mixture of 0, 0.2, 0.5, 1, 2, 3, 4, or 5 µg of the GLI3 expression plasmid pSRa-GLI3, 3 µg of the luciferase reporter plasmid phR-luc, which contains the 3.5-kilobase HindIII-EcoRI fragment of Gli1 promoter, and 0.5 µg of the internal control plasmid pRL-SV, in which the SV40 early promoter was linked to the sea pansy luciferase gene, was transfected into NIH3T3 cells, and luciferase assays were performed. The total amount of plasmid DNA was adjusted to 8.5 µg by adding the control plasmid DNA pSRa0. The experiments were repeated three times, and the differences between the experiments were no more than 20%. Typical results are indicated by a bar graph. The shaded and open bars indicate the data with and without the GLI3 expression plasmid, respectively. D, Shh-induced activation of the Gli1 promoter by GLI3 in MNS-70 cells. A mixture of 0, 0.5, 1, or 2 µg of the Shh expression plasmid pJT4/Shh, 3 µg of the Gli1 wild type promoter-containing luciferase reporter plasmid phR-luc, 3 µg of the GLI3 expression plasmid pSRa-GLI3, 1 µg of the PKA catalytic subunit expression plasmid pSRa-PKA, and 0.5 µg of the internal control plasmid pRL-SV was transfected into MNS-70 cells, and luciferase assays were performed. The total amount of plasmid DNA was adjusted to 8.5 µg by adding the control plasmid DNA. The experiments were repeated three times, and the average is shown as the relative luciferase activity with S.D. E, requirement of GLI3-binding sites for Shh-induced activation of the Gli1 promoter. Co-transfection experiments were done as described in D using the reporter containing the mutant Gli1 promoter in which the eight GLI3-binding sites were mutated.
CBP-binding domain of GLI3 really mediates Shh-induced activation of the Gli1 promoter, the GLI3-binding domain of CBP should mask the CBP-binding domain of GLI3 and inhibit the induction of the endogenous Gli1 gene by Shh. As reported by Sasaki et al. (38), transfection of the Shh expression plasmid increased the level of Gli1 mRNA by about 6.3-fold. Co-transfection of the Shh expression plasmid with the GLI3 expression plasmid strongly enhanced the Gli1 mRNA level by 10.5-fold. In contrast, co-expression of the GLI3-binding domain of CBP with Shh significantly lowered the level of induction of Gli1 mRNA by about half. These results further confirm that GLI3 is a mediator of Shh-dependent transcriptional activation of Gli1.

**DISCUSSION**

**Distinct Roles for GLI3 and Gli1 in Shh Signaling**—In Drosophila, Hh signaling inhibits association of the Ci-Cos2-Fu-Su(fu) complex with the cytoskeleton and cleavage of Ci into a repressor form on the A-P border (18, 19, 25, 11). Although the pathway of Shh signaling has not yet defined completely, there is evidence to suggest that it is well conserved between Drosophila and vertebrates (Fig. 7). First, GLI3, like Ci, is primarily localized in the cytosol (34). Second, GLI3 has a domain structure similar to that of Ci, with both proteins having transcriptional activation and repression domains. Third, GLI3, like Ci, appears to be cleaved into a repressor form. These facts suggest that Shh signaling blocks association of the GLI3-Cos2-Fu-Su(fu) complex to the cytoskeleton and processing of GLI3 into a repressor form. Recently, PKA was demonstrated to directly phosphorylate the multiple sites adjacent to the dCBP-binding domain in Ci protein and to enhance the proteolysis of Ci (56). The multiple phosphorylation sites for PKA are well conserved in GLI3, and our results indicate that the small segment of GLI3 containing the CBP-binding domain and the putative PKA phosphorylation sites is sufficient for positive regulation by Shh and negative regulation by PKA (Fig. 2). In addition, PKA enhances the proteolysis of GLI3 to generate a repressor form, and Shh signaling inhibits this (Fig. 4). Phosphorylation of GLI3 by PKA does not affect binding to CBP. One possibility is that direct phosphorylation of GLI3 by PKA retains GLI3 as part of the cytoskeleton complex in the cytosol and enhances the proteolysis of GLI3. Interestingly, in the absence of PKA, Shh does not enhance trans-activation by Gal4-CBD (Fig. 2E, right panel), suggesting that the role of Shh is to negate the action of PKA. This is consistent with the previous report that suppression of PKA activity is sufficient to activate targets of the Shh signaling pathway in the mouse central nervous system (28). However, the Shh responsiveness of full-length GLI3 in the absence of PKA (Fig. 2E) may suggest that Shh may enhance GLI3 activity through not only the CBP-binding domain but also other region. In addition, Shh and PKA enhanced the Gli1 promoter activity to the higher level than that with Shh alone (Fig. 5D). This may be consistent with the recent report by Ohlmeyer and Kalderon (57) that increased PKA activity can induce ectopic Hh target gene expression without changes in Ci protein concentration. At present, it remains unknown whether CBP is merely act as a co-activator of full-length GLI3 or is important for Shh and PKA responsiveness of GLI3. Because Ci lacks an obvious nuclear localization signal (9, 58), its movement into the nucleus could be mediated by its ability to bind to dCBP, which would carry it there. Although GLI3 has one putative nuclear localization signal in the N-terminal region, vertebrate CBP may also act as a carrier of GLI3 into the nuclei.

Unlike GLI3, Gli1 is a simple transcriptional activator encoded by a target gene of Shh signaling. It appears to contain only the transcriptional activation domain and is not cleaved. These facts suggest the following cascade between Shh and the induction of its target genes (Fig. 7). Shh first enhances Gli3 activity via the region containing the CBP-binding domain, and then the activated GLI3 directly binds to the Gli1 promoter and induces Gli1 transcription. This leads to accumulation of Gli1, which then induces a second wave of transcription involving Shh target genes such as Hnf-3β and possibly the Gli1 gene itself, because Gli1 can activate the Gli1 promoter in co-transfection assays (data not shown).

We observed that the level of Gli1 mRNA in various regions of GLI3 heterozygous and homozygous mouse embryos (Xt/+ and Xt/Xt) was significantly lower than that of the wild type (data not shown). However, Gli1 mRNA did not completely disappear, even in the Gli3 homozygous mutant. This may be due to a redundant function of GLI3 and Gli2. Redundant functions of GLI3 and Gli2 was suggested by a study of Gli2 and Gli3 double mutant mice (59). In MNS-70 cells, Gli1 mRNA is expressed at very low level, although a significant level of Gli3 and Gli2 mRNA expression is still observed (data not shown). Because overexpression of the CBP fragment containing the GLI3-binding domain significantly lowered the Shh-dependent induction of Gli1 mRNA, CBP may also act as a co-activator of GLI2. Gli3 is not thought to be expressed in the ventral midline of the central nervous system, where the floor plate is induced by Shh. In this region, GLI2 may play the same role as GLI3 to induce Gli1 expression. This is consistent with the recent study of Gli2 mutant mice, in which Gli1 is not detected ventrally at E9.5 and the floor plate does not form (60). Recently, it was reported that Gli2 and Gli3 repress the ectopic induction of frog floor plate cells by Gli1 in co-injection assays and inhibit endogenous floor plate differentiation (61). However, a large amount of repressor form of GLI3 and only a small amount of full-length activator form could be generated in this system, because Shh signaling may not act on large amount of GLI3 protein generated from injected mRNA. If this is a case, a repressor form of GLI3 may inhibit the expression of Shh target genes and antagonize Gli1 function. The antagonizing activities of Gli2 and Gli3 on Gli1 function are also not consistent with the recent loss-of-function studies with Gli1 and Gli2 mutants in mouse (60).

**Relationship between Gli3 Mutations and Genetic Diseases**—Mutations and translocations of the human GLI3 gene are responsible for two human disorders, GCPS and PHS (40, 41). The characteristics of each of these disorders overlap with one another but still remain sufficiently distinct to be classified as
Hnf-3 activates specific target genes including Gli1 and induces Hnf-3β and the Gli1 gene itself in an Shh-independent manner.

separate disorders. For instance, although both GCPS and PHS have polysyndactyly and abnormal craniofacial features, GCPS has commonly postaxial polydactyly of the hands and preaxial polydactyly of the feet, whereas PHS has typically central or postaxial polydactyly. GCPS does not cause the hypothalamic hamartoma observed in PHS. In addition, PHS is not associated with hypertelorism or broadening of the nasal root or forehead seen in GCPS. The different anomalies in these two disorders may be partly due to the generation of truncated proteins having different characteristics. In GCPS, GLI3 was found to be truncated upstream or within the zinc finger domain (40, 62), whereas mutations found in PHS truncate GLI3 after the zinc finger (41). Our domain analysis indicated that the N-terminal region upstream from the zinc finger region is a repressor domain. Therefore, the truncated protein found in PHS could still retain repressor activity, unlike the protein truncated upstream or within the zinc finger domain, which was found in GCPS.

**Direct Binding of CBP to GLI3: a Molecular Link between RTS and Genetic Diseases caused by Gli3 Mutations**—Our results indicate that GLI3 utilizes CBP as a co-activator, as in *Drosophila*, in which Ci uses dCBP. Thus, the interaction of GLI3/Ci with CBP/dCBP is conserved between mammals and insects. Some of the characteristics of GCPS and PHS are similar to RTS. GCPS and RTS are caused by haploinsufficiency, and GCPS, PHS, and RTS are all associated with craniofacial, hand, and foot defects. Thus, direct interaction between GLI3 and CBP could explain the similar characteristics of these disorders. However, some characteristics of these syndromes are distinct. For instance, broad thumbs and broad halluces are associated with both GCPS and RTS, but polydactyly and syndactyly are commonly observed only in GCPS. These differences between GCPS and RTS could be explained by multiple mechanisms. In the embryos of multiple polydactylous mouse mutants, such as *Xt* and *Hemimelic extra toes* (*Hx*), Shh is ectopically expressed at the anterior margin of the limb buds (83). By analogy with the Ci-dependent repression of *hh* expression in the anterior compartment of *Drosophila*, the repressor form of GLI3 lacking the C-terminal activation domains may repress Shh expression. Therefore, the reduction or loss of repressor activity of GLI3 may lead to polydactyly. Although the truncated forms found in PHS still retain the N-terminal repressor domain and the zinc finger domain, these truncations may lead to increased instability of these proteins or to lower repressor activity. In contrast, the deficiency of CBP should not affect the repressor activity of GLI3, and this may explain the lack of polydactyly in RTS. In addition, CBP affects the activity of many transcription factors, and some of the features seen in RTS could be explained by decreased activity of transcription factors other than GLI3. dCBP is a co-activator of Dorsal, a *Drosophila* homolog of NF-κB, and dCBP mutations cause the loss of Dorsal-dependent expression of the *twist* gene (64). Because mutations of the human *Twist* gene are associated with the autosomal dominant Saethre-Chotzen syndrome, which is clinically analogous to RTS (65, 66), some defects may be caused by decreased expression of the *Twist* gene.

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**REFERENCES**

1. Ingham, P. W. (1995) *EMBO J.* 17, 3505–3511
2. Ruiz i Altaba, A. (1997) *Cell* 90, 193–196
3. Lee, J. J., von-Roessler, D. P., Parkes, S., and Beachy, P. A. (1992) *Cell* 71, 33–50
4. Herberline, U., Wolff, T., and Rubin, G. M. (1993) *Cell* 75, 914–926
5. Ma, C., Zhou, Y., Beachy, P. A., and Moses, K. (1993) *Cell* 75, 927–938
6. Basler, K., and Struhl, G. (1994) *Nature* 368, 218–221
7. Tabata, T., and Kornberg, T. B. (1994) *Cell* 76, 89–102
8. von Ohlen, T., Lensing, D., Nusse, R., and Hooper, J. E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 2404–2409
9. Orenic, T. V., Slusarski, D. C., Krell, K. L., and Holmgren, R. A. (1990) *Genes Dev.* 4, 1053–1067
10. Alexandre, C., Jacinto, A., and Ingham, P. W. (1996) *Genes Dev.* 10, 2003–2013
11. Aza-Balnc, P., Ramirez-Weber, F.-A., Laget, M.-P., Schwartz, C., and Kornberg, T. B. (1997) *Cell* 89, 1043–1053
12. Domínguez, M., Brunner, M., Hafend, E., and Basler, K. (1996) *Science* 272, 1621–1625
13. Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J. E. (1996) *Cell* 86, 221–232
14. Chalfie, M., and Struhl, G. (1996) *Cell* 87, 553–563
15. Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M., and Tabin, C. J. (1996) *Nature* 384, 176–179
16. Stone, D. M., Hynes, M., Amanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Philips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996) *Nature* 384, 129–134
17. Preat, T., Thérond, P., Lamour-Issard, C., Limbourg-Bouchon, B., Tricoire, H., Erci, I., Marcel, M. C., and Busson, D. (1996) *Nature* 374, 87–89
18. Robbins, D. J., Nyhakken, K. E., Kobayashi, R., Sisson, J. C., Bishop, J. M., and Thérond, P. P. (1997) *Cell* 90, 225–234
19. Sisson, J. C., Ho, K. S., Sayanna, K., and Scott, M. P. (1997) *Cell* 90, 235–245
20. Jiang, J., and Struhl, G. (1995) *Cell* 80, 563–572
21. Lepage, T., Cohen, S. M., Díaz-Benjumea, F. J., and Parkhurst, S. M. (1995) *Nature* 373, 711–715
22. Li, W., Ohlmeyer, J. T., Lane, M. E., and Kalderon, D. (1995) *Cell* 80, 553–562
23. Pan, D., and Rubin, G. M. (1995) *Cell* 80, 543–552
24. Strutt, D. I., Wiersdorff, V., and Mlodzik, M. (1995) *Nature* 373, 705–709
25. Monnier, V., Dussilpol, Alves, G., Lamour-Issard, C., and Pleiss, A. (1998) *Curr. Biol.* 8, 583–586
26. Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996) *Nature* 383, 407–413
27. Hemmingsen, M., Biggsm, M. J., and McMahon, A. P. (1996) *Genes Dev.* 10, 647–658
28. Epstein, D. J., Marti, E., Scott, M. P., and McMahon, A. P. (1996) *Development* 122, 2885–2904

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**Fig. 7. Schematic representation of the role of GLI3 and GLI1 in Shh signaling.** Shh signaling enhances the trans-activating capacity of GLI3 through the region containing the CBP-binding domain and the PKA phosphorylation sites and induces Gli1 transcription through the direct action of GLI3 on the Gli1 promoter. The expressed GLI1 then activates specific target genes including Hnf-3β and the Gli1 gene itself in an Shh-independent manner.
