Integrative genomic analyses on *GLI1*: Positive regulation of GLI1 by Hedgehog-GLI, TGFß-Smads, and RTK-PI3K-AKT signals, and negative regulation of GLI1 by Notch-CSL-HES/HEY, and GPCR-Gs-PKA signals

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Abstract. GLI family members are zinc-finger transcription factors, which are involved in embryogenesis and carcinogenesis through transcription regulation of *GLI1, CCND1, CCND2, FOXA2, FOXC2, RUNX2, SFRP1,* and *JAG2*. *GLI1* transcription is upregulated in a variety of human tumors, such as basal cell carcinoma, lung cancer, breast cancer, gastric cancer, pancreatic cancer, and esophageal cancer. Hedgehog signaling via Smoothened cascade and receptor tyrosine kinase (RTK) signaling via PI3K-AKT cascade induce stabilization of GLI1 protein, whereas G-protein coupled receptor (GPCR) signaling via Gs-PKA cascade induces degradation of GLI1 protein. Here we report integrative genomic analyses of the *GLI1* gene. The *GLI1* and *ARHGAP9* genes are located in a tail-to-tail manner with overlapping 3'-ends. *ARHGAP9* was expressed in bone marrow, spleen, thymus, monocytes, and macrophages, whereas *GLI1* was almost undetectable in normal tissues or cells with predominant *ARHGAP9* expression. Because overlapping sense and anti-sense transcripts are annealed to each other to give rise to double-stranded RNAs functioning as endogenous RNAi, *GLI1* expression might be negatively regulated by *ARHGAP9* transcripts. GLI-binding element with one base substitution at the +1589-bp position from the transcriptional start site (TSS) of the human *GLI1* gene was completely conserved in chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes. Ten Smad-binding elements, double E-boxes for EMT regulators, and double N-boxes for HES/HEY family members within intron 1 of the human *GLI1* gene were also conserved in mammalian *GLI1* orthologs. *GLI1* transcription is upregulated due to Hedgehog, and TGFß signaling activation, whereas *GLI1* transcription is downregulated due to Snail/Slug, and Notch signaling activation. Together these facts indicate that Hedgehog, TGFß, and RTK signals positively regulate GLI1, and that Notch, and GsPCR signals negatively regulate the GLI1.

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Introduction

Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH) are secreted-type glycoproteins with lipid modifications involved in embryogenesis, adult tissue homeostasis, and carcinogenesis (1-4). Mature Hedgehog proteins bind to Patched family receptors to activate Smoothened signaling cascade, which results in GLI-mediated transcriptional upregulation of Hedgehog target genes (5-8).

GLI1, GLI2, and GLI3 are GLI family members (9-14). GLI1 consists of zinc finger domains, and C-terminal activator domain, whereas GLI2 and GLI3 consist of N-terminal repressor domain, zinc finger domains, and C-terminal activator domain. In the absence of Hedgehog signals, GLI1 is transcriptionally repressed, GLI2 is phosphorylated for the FBXW11/ßTRCP2-mediated degradation, and GLI3 is phosphorylated for the FBXW1/ßTRCP1-mediated processing into repressor (10-12). In the presence of Hedgehog signals, stabilized GLI family members induce transcriptional activation of target genes, such as *GLI1, CCND1, CCND2, FOXA2, FOXC2, RUNX2, SFRP1,* and *JAG2* (15-21). Hedgehog-induced *GLI1* upregulation augments the Hedgehog signaling cascades through a positive-feedback mechanism.

GLI1 is upregulated in various types of human tumors, as summarized in Table I. *GLI1* is amplified and overexpressed in glioma (9), rhabdomyosarcoma (22), and osteosarcoma (22). *GLI1* is also amplified in B-cell lymphoma (23). Although *GLI1* is almost undetectable in adult human tissue by using RNase A protection assay, *GLI1* expression is detected in embryonal carcinoma (24). Because *GLI1* is a representative target gene of the Hedgehog signaling cascades, *GLI1* is upregulated in basal cell carcinoma (25), medulloblastoma (26), lung cancer (27), gastric cancer (28,29), pancreatic cancer (28,30), esophageal cancer (28,31), breast cancer (32), prostate

Table I. Genetic alteration or expression of *GLI1* in human tumors.

Human cancer	Expression and mechanism	Refs.
Glioma	Gene amplification and overexpression	(9)
Rhabdomyosarcoma	Gene amplification and overexpression	(22)
Osteosarcoma	Gene amplification and overexpression	(22)
B-cell lymphoma	Gene amplification	(23)
Embryonal carcinoma	Expression	(24)
Basal cell carcinoma	Upregulation by Hedgehog signaling	(25)
Medulloblastoma	Upregulation by Hedgehog signaling	(26)
Lung cancer	Upregulation by Hedgehog signaling	(27)
Gastric cancer	Upregulation by Hedgehog signaling	(28,29)
Pancreatic cancer	Upregulation by Hedgehog signaling	(28,30)
Esophageal cancer	Upregulation by Hedgehog signaling	(28,31)
Breast cancer	Upregulation by Hedgehog signaling	(32)
Prostate cancer	Upregulation by Hedgehog signaling	(33)
Liver cancer	Upregulation by Hedgehog signaling	(34)
Cervical cancer	Upregulation by Hedgehog signaling	(35)
Endometrial cancer	Upregulation by Hedgehog signaling	(36)
Melanoma	Upregulation by Hedgehog signaling	(37)
Ovarian cancer	Upregulation by Hedgehog signaling	(38)
Ewing sarcoma	Upregulation by EWS/FLI1 fusion protein	(39)

cancer (33), liver cancer (34), cervical uterine cancer (35), endometrial uterine cancer (36), melanoma (37), and ovarian cancer (38). *GLI1* is also upregulated based on the EWS-FLI fusion protein generated by chromosomal translocation in Ewing sarcoma (39).

The *GLI1* core promoter, defined as a region surrounding the transcription start site (TSS), is involved in the transcriptional regulation of human *GLI1* gene (40). Hedgehog signals induce transcriptional upregulation of mouse *Gli1* by using GLI-binding site within intron 1 (41), whereas TWIST1/TWIST induces transcriptional upregulation of human *GLI1* by using E-boxes within intron 1 (42). Because proximal promoter region and intron 1 are involved in transcriptional regulation of *GLI1*, we carried out refined integrative genomic analyses of *GLI1* to elucidate the mechanisms of GLI1 upregulation in human tumors with the emphasis on the stem-cell signaling network.

Materials and methods

Comparative genomic analyses. Human genome sequences corresponding to human GLI1 RefSeq (NM_005269.2) were searched for by using BLAST programs, as previously described (43,44). *GLI1* expressed sequence tags (ESTs) were also searched for to identify *GLI1* splicing variants (45,46). Conserved transcription factor-binding sites within *GLI1* promoters were then searched for based on manual inspection, as previously described (47,48).

Regulatory network analyses. The literature on Hedgehog, TGFß, Notch and receptor tyrosine kinase (RTK) signaling cascades in PubMed and Medline databases was critically evaluated to extract knowledge. The mechanisms of *GLI1* transcription were then investigated based on our data of conserved transcription factor-binding sites within *GLI1* regulatory regions and in-house knowledgebase of transcription factors regulated by the stem-cell signaling network.

Results

Comparative genomic analyses of GLI1. BLAST programs using GLI1 RefSeq (NM_005269.2) as a query sequence revealed that *GLI1* gene is located within human genome sequence AC022506.38. Human *GLI1* gene, consisting of 12 exons, is about 12 kb in size, as previously reported by Liu *et al* (40). Human *GLI1* gene is located between the *INHBC-INHBE* gene cluster and the *ARHGAP9* gene at human chromosome 12q13.3. The *GLI1* gene is the paralog of the *GLI2* gene at human chromosome 2q14.12, whereas the *INHBC* and *INHBE* genes encoding Inhibin family members are paralogs of the *INHBB* gene neighboring the *GLI2* gene (data not shown). The *INHBC-INHBE-GLI1* locus at human chromosome 12q13.3 and the *INHBB-GLI2* locus at human chromosome 2q14.12 are paralogous regions within the human genome.

The *GLI1* and *ARHGAP9* genes are located in a tail-to-tail manner with the overlapping 16 bases in their 3'-ends (Fig. 1). *ARHGAP9* is expressed in peripheral blood leukocytes, spleen, thymus (49), and also in thyroid gland, testis, bone marrow, monocytes, and macrophages (RefEX database). *GLI1* is almost undetectable in normal tissues with predominant *ARHGAP9* expression (RefEX database). Because overlapping sense and anti-sense transcripts are annealed to

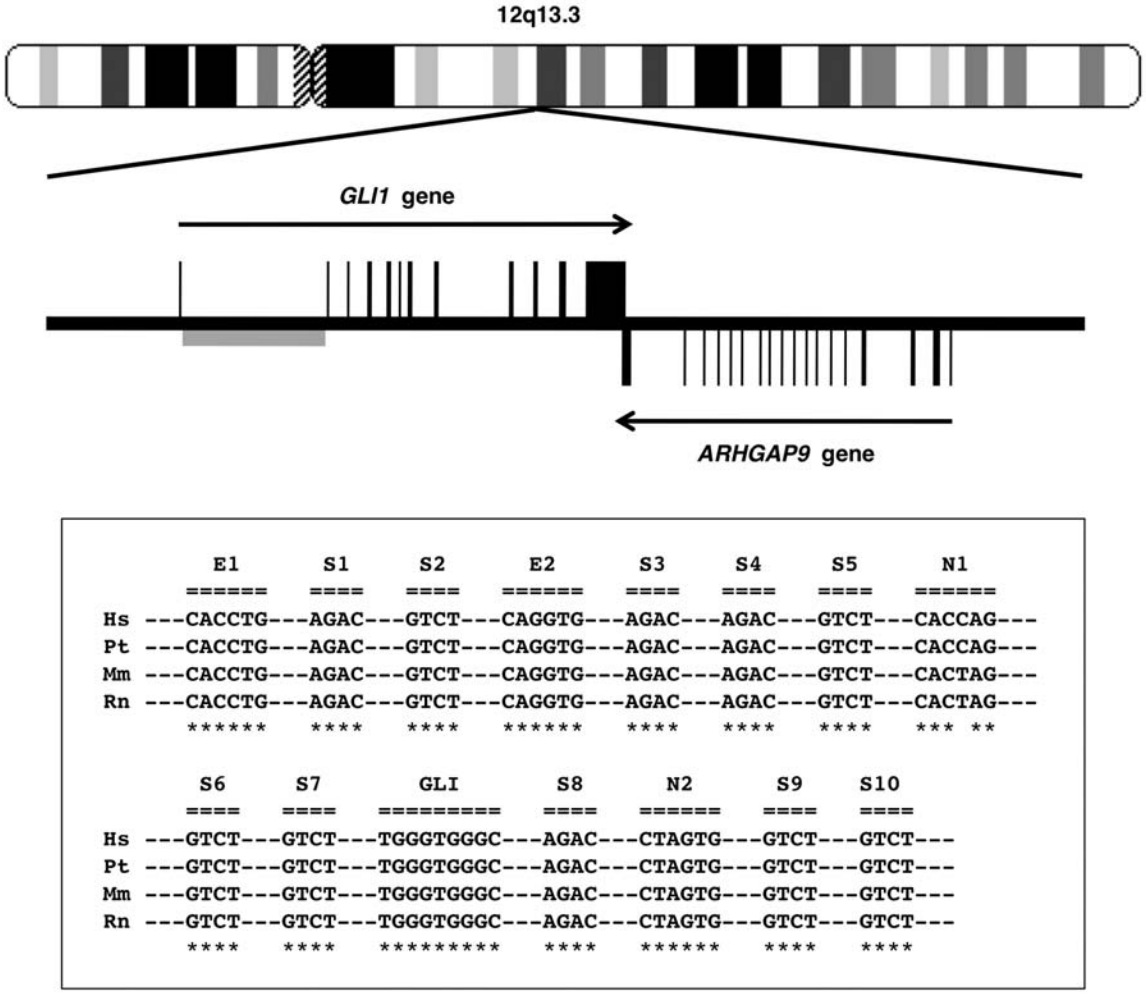

Figure 1. Integrative genomic analyses of *GLI1*. Schematic representation of *GLI1* and *ARHGAP9* genes at human chromosome 12q13.3 is shown in the upper part. *GLI1* gene consisting of 12 exons and *ARHGAP9* gene consisting of 18 exons are located in a tail-to-tail manner with overlapping 3'-ends. Conserved transcription factor-binding sites within intron 1 of *GLI1* gene are shown in the gray box. E-boxes (E1 and E2), N-boxes (N1 and N2), Smad-binding elements (S1-S10), and GLI-binding site (GLI) are shown. Hs, human; Pt, chimpanzee; Mm, mouse; Rn, rat.

each other to give rise to double-stranded RNAs functioning as endogenous RNAi, *GLI1* expression might be negatively regulated by the *ARHGAP9* transcripts.

Comparative genomic analyses of GLI1 orthologs. BLAST programs using human *GLI1* genome sequence as a query sequence revealed that chimpanzee *GLI1* gene, mouse *Gli1* gene, and rat *Gli1* gene were located within NW_001223153.1, AC114678.20, and AC122965.5 genome sequences, respectively. *GLI1* orthologs were well conserved not only within exonic regions, but also within the proximal promoter region and intron 1 (data not shown).

Consensus GLI-binding element within the regulatory regions of human *GLI1* gene was searched for based on manual inspection, and a GLI-binding element with one base substitution was identified at the +1589-bp position from the TSS (Fig. 1). The GLI-binding element was completely conserved in intron 1 of chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes (Fig. 1). Vokes *et al* carried out chromatin-immunoprecipitation assay combined with microarray analyses (ChIP-chip assay) of GLI-binding sites in mouse embryonic stem (ES) cells, and showed that the major peak of GLI-binding site within the regulatory regions of mouse *Gli1* gene was located within intron 1 (41). We confirmed that the conserved GLI-binding element identified in this study was located within the genome fragment immunoprecipitated by using the anti-Gli1 antibody (data not shown). Therefore, it was concluded that the GLI-binding site at about +1.5-kb position from the TSS was evolutionarily conserved among mammalian *GLI1* orthologs (Fig. 1).

Smad-binding element (SBE) within the regulatory regions of human *GLI1* gene was next searched for based on manual inspection, and 10 SBEs within intron 1 of human *GLI1* gene were found to be conserved in mouse *Gli1* gene (Fig. 1). These 10 SBEs were also conserved in chimpanzee *GLI1* and rat *Gli1* genes (Fig. 1). TGFß signals induce Smad3-dependent upregulation of *GLI1* and *GLI2* in human NHDF fibroblasts, HaCaT keratinocytes, and MDA-MB-231 breast cancer cells (50); however precise mechanisms of Smad3-mediated *GLI1* upregulation remained unclear. Conserved SBEs within intron 1 of mammalian *GLI1* ortholgs clearly elucidated the mechanism of TGFß-induced *GLI1* upregulation (Fig. 2).

Six bHLH-binding sites within intron 1 of human *GLI1* gene were conserved in chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes (data not shown). Two bHLH-binding sites located within the 5'-region of intron 1 (Fig. 1) were the specific

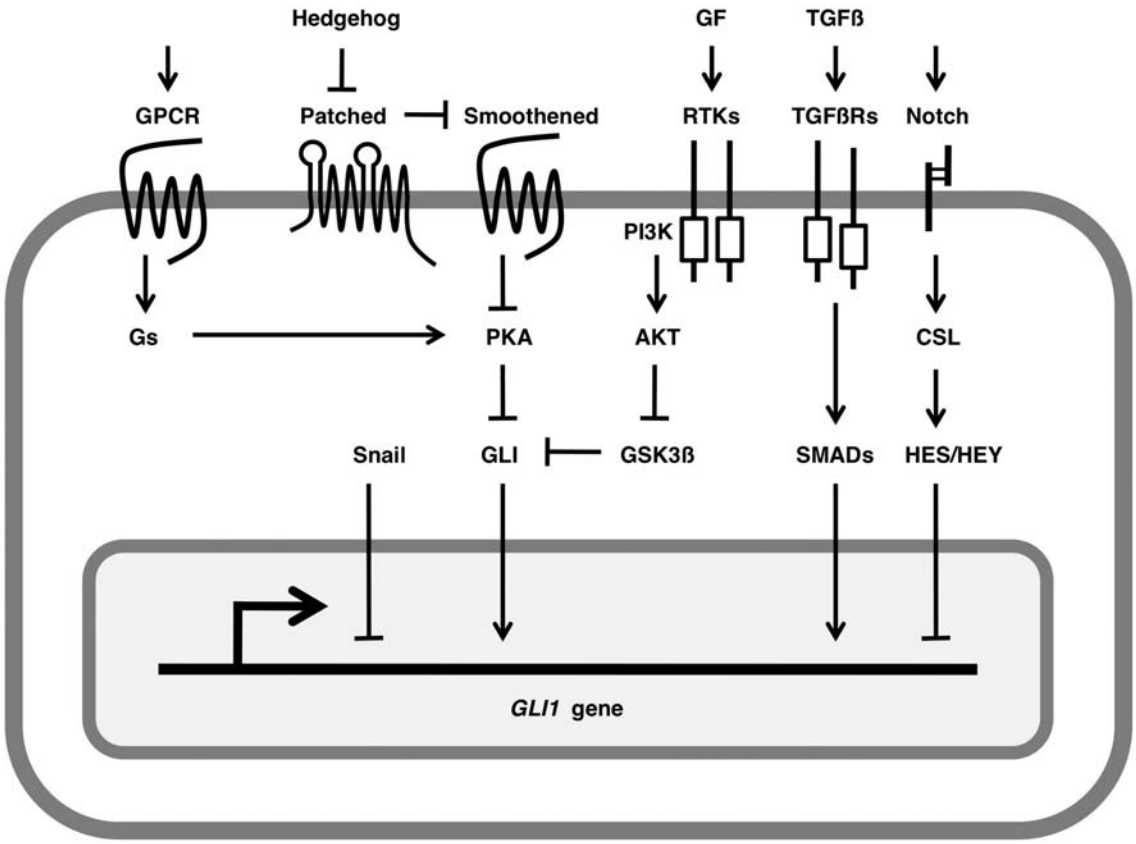

Figure 2. Stem-cell signaling network and GLI1 regulation. GLI1 is activated due to transcriptional activation as well as protein stabilization. Hedgehog and TGFß signals induce transcriptional upregulation of *GLI1*, whereas Notch signals induce transcriptional downregulation of *GLI1*. Receptor tyrosine kinase (RTK) signals via PI3K-AKT signaling cascade induce stabilization of GLI1 protein, whereas G-protein coupled receptor (GPCR) signals via Gs-PKA signaling cascade induce degradation of GLI1 protein.

E-boxes utilized as binding sites for TWIST1 (42). SNAI1/Snail, SNAI2/Slug, ZEB1/δEF1/ZFHX1A, ZEB2/SIP1/ZFHX1B, and TWIST2/DERMO1 also bind to the same E-boxes as TWIST1 to regulate the transcription of target genes (51-54). ZEB1, ZEB2, TWIST1, and TWIST2 function as transcriptional activators or repressors in a context-dependent manner, whereas SNAI1 and SNAI2 function as transcriptional repressors. Based on these facts, it was predicted that SNAI1 and SNAI2 repress *GLI1* expression via the E-boxes (Fig. 2).

Double N-boxes within intron 1 of human *GLI1* gene were also conserved in chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes (Fig. 1). Notch ligands induce Notch signaling activation through the NICD-CSL complex to upregulate Notch target genes, such as *HES1, HES5, HEY1, HEY2* and *HEYL* (55-58). Consensus CSL-binding site was not conserved in mammalian *GLI1* orthologs, whereas double N-boxes utilized as HES/HEY-binging sites within intron 1 of human *GLI1* gene were conserved in chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes (Fig. 1). Together these facts indicate that Notch signals downregulate *GLI1* expression via HES/HEY family members (Fig. 2).

Discussion

Integrative genomic analyses of *GLI1* gene were carried out in this study. The *GLI1* and *ARHGAP9* genes are located in a tail-to-tail manner with overlapping 3'-ends (Fig. 1). *ARHGAP9* was expressed in bone marrow, spleen, thymus, monocytes, and macrophages, whereas *GLI1* was almost undetectable in normal hematopoietic tissues or cells with predominant *ARHGAP9* expression. Because overlapping sense and anti-sense transcripts are annealed to each other to give rise to double-stranded RNAs functioning as endogenous RNAi, *GLI1* expression might be negatively regulated by *ARHGAP9* transcripts.

GLI-binding element with one base substitution at the +1589-bp position from the TSS of human *GLI1* gene was completely conserved in chimpanzee *GLI1*, mouse *Gli1*, and rat *Gli1* genes (Fig. 1). Ten Smad-binding elements, double E-boxes and double N-boxes within intron 1 of human *GLI1* gene were also conserved in mammalian *GLI1* orthologs (Fig. 1). Hedgehog and TGFß signals induce transcriptional upregulation of target genes via GLI and Smads, respectively (Fig. 2). On the other hands, Snail/Slug and Notch-HES/HEY signals induce transcriptional downregulation of target genes via E-box and N-box, respectively (Fig. 2). *GLI1* transcription is upregulated due to Hedgehog, and TGFß signaling activation, whereas *GLI1* transcription is downregulated due to Snail/Slug, and Notch signaling activation.

GLI1 is activated based on transcriptional activation as well as protein stabilization (Fig. 2). Hedgehog and TGFß signals induce transcriptional upregulation of *GLI1*, whereas Notch signals induce transcriptional downregulation of *GLI1*. RTK signals via the PI3K-AKT signaling cascade induce stabilization of GLI1 protein, whereas G-protein coupled

receptor (GPCR) signals via the Gs-PKA signaling cascade induce degradation of the GLI1 protein. Together these facts indicate that Hedgehog, TGFß, and RTK signals positively regulate GLI1, and that Notch, and GsPCR signals negatively regulate the GLI1 (Fig. 2).

Hedgehog, TGFß/BMP, RTK, Notch, and WNT signaling cascades constitute the stem-cell signaling network to orchestrate embryogenesis and carcinogenesis (59-63). Dysregulation of the stem-cell signaling network leads to GLI1 upregulation to promote carcinogenesis (Fig. 2). KAAD-cyclopamine and HhAntag are small-molecule compounds indirectly targeted to GLI1 and GLI2 via Smoothened (64,65), whereas GANT61 is a small-molecule compound directly targeted to GLI1 and GLI2 (66). GLI-targeted small-molecule compounds could show anti-tumor effects to a broader range of cancer than Smoothened-targeted small-molecule compounds. To realize personalized medicine in the sequencing era with a peta-scale supercomputer (67), development of GANT61-like compounds for clinical application is expected.

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