Integrative genomic analyses on GLI2: Mechanism of Hedgehog priming through basal GLI2 expression, and interaction map of stem cell signaling network with P53

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Abstract. Hedgehog-binding to Patched family receptors results in Smoothened-mediated activation of MAP3K10 (MST) and inactivation of SUFU. MAP3K10-induced DYRK2 phosphorylation combined with SUFU inhibition results in the stabilization and nuclear accumulation of GLI2 for transcriptional activation of *GLI1, CCND1, CCND2, FOXA2, FOXC2, FOXP3, FOXQ1, RUNX2*, and *JAG2*. Here, integrative genomic analyses on GLI2 orthologs were carried out. Rat Gli2 complete coding sequence was determined by assembling nucleotide sequences of exons 1, 2, and 5'-truncated rat Gli2 RefSeq (NM_001107169.1). GLI2 orthologs were more related to GLI3 orthologs than to GLI1 orthologs lacking the N-terminal repressor domain. ßTRCP1 (FBXW1)-binding DSYxxxS motif was conserved in GLI2 and GLI3 orthologs, while ßTRCP2 (FBXW11)-binding DSGxxxxxxxxS motif in GLI2 and GLI1 orthologs. Human *GLI2* mRNA was expressed in ES cells, NT2 cells, fetal lung, fetal heart, regenerating liver, gastric cancer, and other tumors. Mouse *Gli2* mRNA was expressed in unfertilized egg, ES cells, and EG cells. Tandem RRRCWWGYYY motifs for P53, P63 or P73, and also four conserved bHLH-binding sites were identified within *GLI2* proximal promoter region. Interaction map of P53 and stem cell signaling network were then constructed. P53-induced *NOTCH1* upregulation leads to *HES1, HES5, HEY1, HEY2* or *HEYL* upregulation for the repression of tissue specific bHLH transcriptional activators. DYRK2 functions as a positive regulator of P53-mediated apoptosis, and also as a negative regulator of the Hedgehog signaling cascade. GLI2 expression is regulated based on the balance of P53, Notch, and TGF-ß signaling, and Hedgehog signaling activation results in cell survival and proliferation due to transcriptional activation of Hedgehog-target genes, and also partly due to perturbation of P53-mediated transcriptional regulation.

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Introduction

Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH) are Hedgehog family members, regulating embryogenesis, and adult tissue homeostasis, and carcinogenesis (1-5). Hedgehog signaling is activated in various human tumors, such as basal cell carcinoma, melanoma, small cell lung cancer, esophageal cancer, gastric cancer, pancreatic cancer, and malignant lymphoma (6-14).

Hedgehog precursors are processed to cut off the C-, and N-terminal regions for cholesteroylation and palmitoylation, respectively (15,16). Mature Hedgehog proteins are then transported to the cell surface for DISP1-dependent packaging into lipoprotein particles, or for lipophilic tail-mediated multimerization (16,17). PTCH1 and PTCH2 are Hedgehog receptors, while CDON, BOC, and GAS1 are Hedgehog co-receptors (6,18-20). Hedgehog-binding to Patched family receptors results in Smoothened signaling activation due to the Hedgehog-mediated internalization of Patched family receptors suppressing Smoothened function (21,22). Hedgehog-induced Smoothened activation leads to MAP3K10 (MST) activation and SUFU inactivation (23,24).

GLI1, GLI2, and GLI3 constitute the GLI family of transcription factors (25,26). GLI1 consists of zinc finger domains, and C-terminal activator domain. On the other hand, GLI2 and GLI3 consist of N-terminal repressor domain, zinc finger domains, and C-terminal activator domain. In the absence of Hedgehog-induced Smoothened activation, GLI1 is transcriptionally repressed, GLI2 is phosphorylated for the ßTRCP2 (FBXW11)-mediated degradation, and GLI3 is phosphorylated for the ßTRCP1 (FBXW1)-mediated processing into repressor lacking the C-terminal activator domain (26-28). In the presence of Hedgehog-induced Smoothened activation, MAP3K10 activation and SUFU inactivation leads to the stabilization and nuclear translocation of GLI2, which results in transcriptional activation of target genes, including *GLI1, CCND1, CCND2, FOXA2, FOXC2, FOXP3, FOXQ1, RUNX2,* and *JAG2* (29,30). GLI1 upregulation augments the Hedgehog signaling cascades through positive-feedback mechanism,

while Cyclin D upregulation promotes cellular proliferation. Therefore, GLI2 is the key molecule implicated in the initial activation of the Hedgehog signaling cascades.

Because transcriptional mechanism of *GLI2* remains almost unclear, we carried out integrative genomic analyses on GLI2 orthologs. Complete coding sequence of rat Gli2 was determined for the comparative proteomic analyses. Complete tandem P53-binding sites and four bHLH-binding sites were next identified within proximal promoter region of human *GLI2* promoter. Then, interaction map of GLI2 and P53 were constructed with the emphasis on the stem cell signaling network.

Materials and methods

Comparative genomic analyses. Rat genome sequences homologous to human GLI2 and mouse Gli2 were searched for by using the BLAST programs as previously described (31,32). Conserved transcription factor-binding sites within promoter regions were then searched for based on the Match program, and manual curation as previously described (33,34).

Comparative proteomic analyses. CLUSTALW program was used for phylogenetic analysis on human and rodent GLI family members. Amino-acid sequences of human GLI1 (NP_005260.1), GLI2 (NP_005261.2), GLI3 (NP_000159.3), mouse Gli1 (NP_034426.2), Gli2 (NP_001074594.1), Gli3 (NP_032156.2), and rat Gli2 (Fig. 1) were used for the phylogenetic analysis.

In silico expression analyses. Expressed sequence tags (ESTs) derived from human *GLI2* and mouse *Gli2* were searched for using the BLAST programs as previously described (35,36). Human GLI2 RefSeq (NM_005270.3) and mouse Gli2 RefSeq (NM_001081125.1) were used as query sequences for the BLAST programs.

Results

Complete coding sequence of rat Gli2. Preliminary phylogenetic analyses on the mammalian GLI family members revealed that rat Gli2 protein RefSeq (NP_001100639.1) deduced from rat Gli2 nucleotide RefSeq (NM_001107169.1) was N-terminally truncated. Comparative genomic analyses next revealed that the 5'-truncation of NM_001107169.1 RefSeq was due to the lack of nucleotide sequences corresponding to exons 1 and 2 of rat *Gli2* gene. Therefore, we decided to determine the complete coding sequence of rat *Gli2* transcript in this study.

BLAST programs revealed that missing exons 1 and 2 of the rat *Gli2* gene were located within rat genome sequence AC120834.4. Exon 1 corresponded to the complementary sequence for nucleotide position 200441-200128 of AC120834.4, and exon 2 to the complementary sequence for nucleotide position 151596-151421. Complete coding sequence of rat Gli2 was then determined by assembling nucleotide sequences of exons 1, 2, and 5'-truncated RefSeq NM_001107169.1. Because nucleotide position 346-4980 was the coding region of rat Gli2 complete coding sequence, rat *Gli2* gene was found to encode 1544 amino-acid Gli2 protein (Fig. 1A).

Comparative proteomics on mammalian GLI family members. Refined phylogenetic analysis on the GLI family members using the full-length rat Gli2 protein revealed that GLI2 orthologs were more related to GLI3 orthologs than to GLI1 orthologs (Fig. 1B). Alignment of mammalian GLI family members next revealed that GLI1 orthologs were shorter than GLI2 and GLI3 orthologs due to the lack of the N-terminal repressor domain.

Bhatia *et al* reported that DSGxxxxxxxxS motif in GLI2 orthologs is the ßTRCP2-binding site to induce degradation (27), and Tempé *et al* reported that DSYxxxS motif in Gli3 is the ßTRCP1-binding site to induce proteolysis (28). We found that the DSGxxxxxxxxS motif was conserved in GLI2 and GLI1 orthologs, and also that the DSYxxxS motif was conserved in GLI2 and GLI3 orthologs (Fig. 1C and D).

Expression profile of GLI2 orthologs. Human *GLI2* mRNA was expressed in ES cells, NT2 cells, fetal lung, fetal heart, regenerating liver, gastric cancer, ovarian cancer, prostate cancer, acute myelogenous leukemia, neuroblastoma, and glioblastoma. Mouse *Gli2* mRNA was expressed in unfertilized egg, ES cells, and EG cells.

Comparative genomics on mammalian GLI2 orthologs. Because the nucleotide sequence of proximal promoter region of rat *Gli2* gene was homologous to 5'-region of human *GLI2* exon 1a rather than that of exon 1b, comparative genomic analyses were focused on the 5'-region of human *GLI2* exon 1a in this study.

Transcription factor-binding sites within the 5'-region of human *GLI2* exon 1a were then searched for. Although it was previously reported that TGFß induces Smad3-mediated *GLI2* upregulation in human fibroblasts, keratinocytes, and various cancer cell lines (37), Smad3-binding site within the 5'-region of human *GLI2* exon 1a was not successfully identified due to the too redundant sequences of Smad-3-binding sites. Instead, basic helix-loop-helix (bHLH)-binding sites, and tandem RRRCWWGYYY motifs without any space for P53, P63 or P73 transcription factors were identified within the 1.5-kb region just upstream of the transcriptional start site of human *GLI2* exon 1a (Fig. 2A). Four basic helix-loop-helix (bHLH)-binding sites within human *GLI2* promoter were conserved in the mouse *Gli2* promoter.

Tandem P53-binding motifs within human *GLI2* promoter were not conserved in rodents due to significant regional divergence, and not in chimpanzee due to C>T nucleotide substitution at the consensus C residue of the first P53-binding motif. Because the tandem P53-binding motifs within the *GLI2* promoter were destroyed in chimpanzee *GLI2* promoter due to single nucleotide substitution, we next searched for a single nucleotide polymorphism (SNP) at that position. However, we could not detect any SNP at the P53-binding site within *GLI2* promoter at least in AC017033.5 and AC016764.8 BAC sequences and personal genome sequences of Dr Venter and Dr Watson.

Together these facts indicate that four bHLH-binding sites within *GLI2* promoter were evolutionarily conserved, and

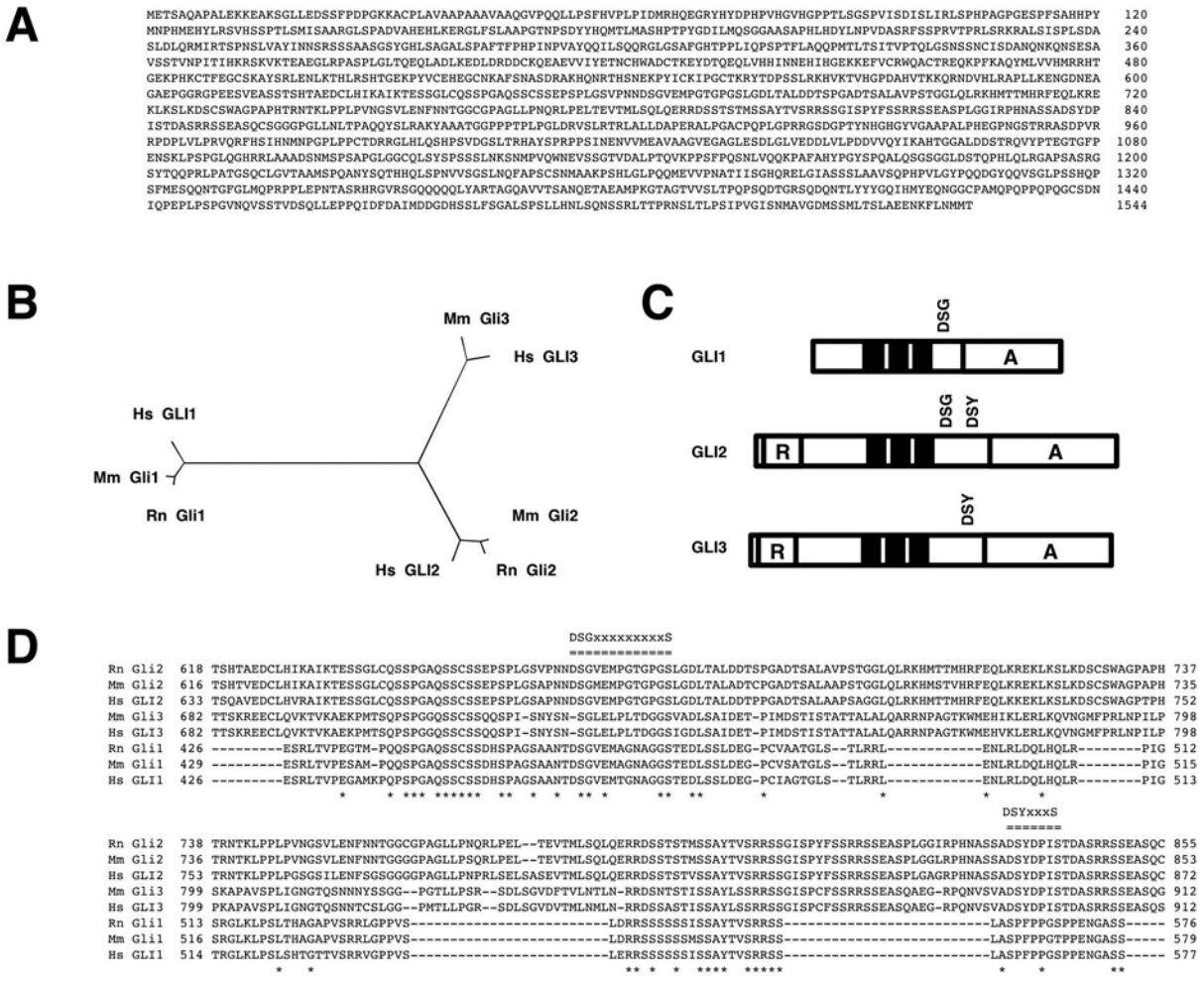

Figure 1. Comparative proteomics on the GLI family. (A), Amino-acid sequence of rat Gli2. (B), Phylogenetic tree of human and rodent GLI family members. (C), Domain architecture of GLI orthologs. R, repressor domain; A, activator domain; DSG, DSGxxxxxxxxxS motif; DSY, DSYxxxS motif; closed boxes, zinc finger domains. (D), Partial alignment of GLI family members around the DSGxxxxxxxxxS and DSYxxxS motifs.

that the tandem P53-binding motifs within the *GLI2* promoter were human specific.

Interaction map of P53 with stem cell signaling network. Hedgehog, BMP/TGFß/Nodal/Activin, EGF/FGF, Notch, and WNT signaling cascades constitute stem cell signaling network (3,38-41). Because both P53 and stem cell signaling network are implicated in carcinogenesis, we next constructed the interaction map of P53 with stem cell signaling network.

P53-induced *Notch1* upregulation (42,43) leads to up-regulation of Hes/Hey family members to repress tissue specific bHLH transcriptional activators (36,44-46). bHLH and P53 are predicted to regulate *GLI2* transcription (Fig. 2A), and TGF-ß signaling to Smad3 is also implicated in GLI2 upregulation as mentioned above (37). Together these facts indicate that GLI2 expression is regulated based on the balance of P53, Notch, and TGF-ß signaling (Fig. 2B).

We cloned and characterized MAP3K10 (MST) as a novel serine/threonine kinase derived from MKN28 gastric cancer cells in 1995 (47), and Taipale's group identified MAP3K10 as a Hedgehog signaling component in 2008 (23). MAP3K10 is reported to directly phosphorylate DYRK2 for its functional inhibition (23). DYRK2 functions as a negative regulator of the Hedgehog signaling cascade (23), but also as a positive regulator of P53-mediated apoptosis (24). GLI2-mediated Hedgehog signaling activation results in cell survival and proliferation due to transcriptional activation of Hedgehog-target genes, and also partly due to perturbation of P53-mediated transcriptional regulation (Fig. 2B).

Discussion

Complete coding sequence of rat Gli2 was determined in this study (Fig. 1A). GLI2 and GLI3 orthologs shared the common domain architecture, consisting of N-terminal repressor domain, zinc finger domains, ßTRCP1-binding DSYxxxS motif, and C-terminal activator domain (Fig. 1C).

Although GLI2 orthologs were more related to GLI3 orthologs than to GLI1 orthologs, ßTRCP2-binding DSGxxxxx xxxxS motif was conserved in GLI2 and GLI1 orthologs, but not in GLI3 orthologs (Fig. 1C). DYRK2, PKA and GSK3ß phosphorylate GLI2 in the absence of Hedgehog signaling (23,48), and phosphorylation of the DSGxxxxxxxxxS motif in GLI2 leads to ßTRCP2-mediated ubiquitination and subsequent proteasome-dependent degradation (27). In the absence of Hedgehog signaling, *GLI1* mRNA is transcriptionally repressed, and GLI1 protein might also be degraded due to the same mechanism as GLI2 degradation.

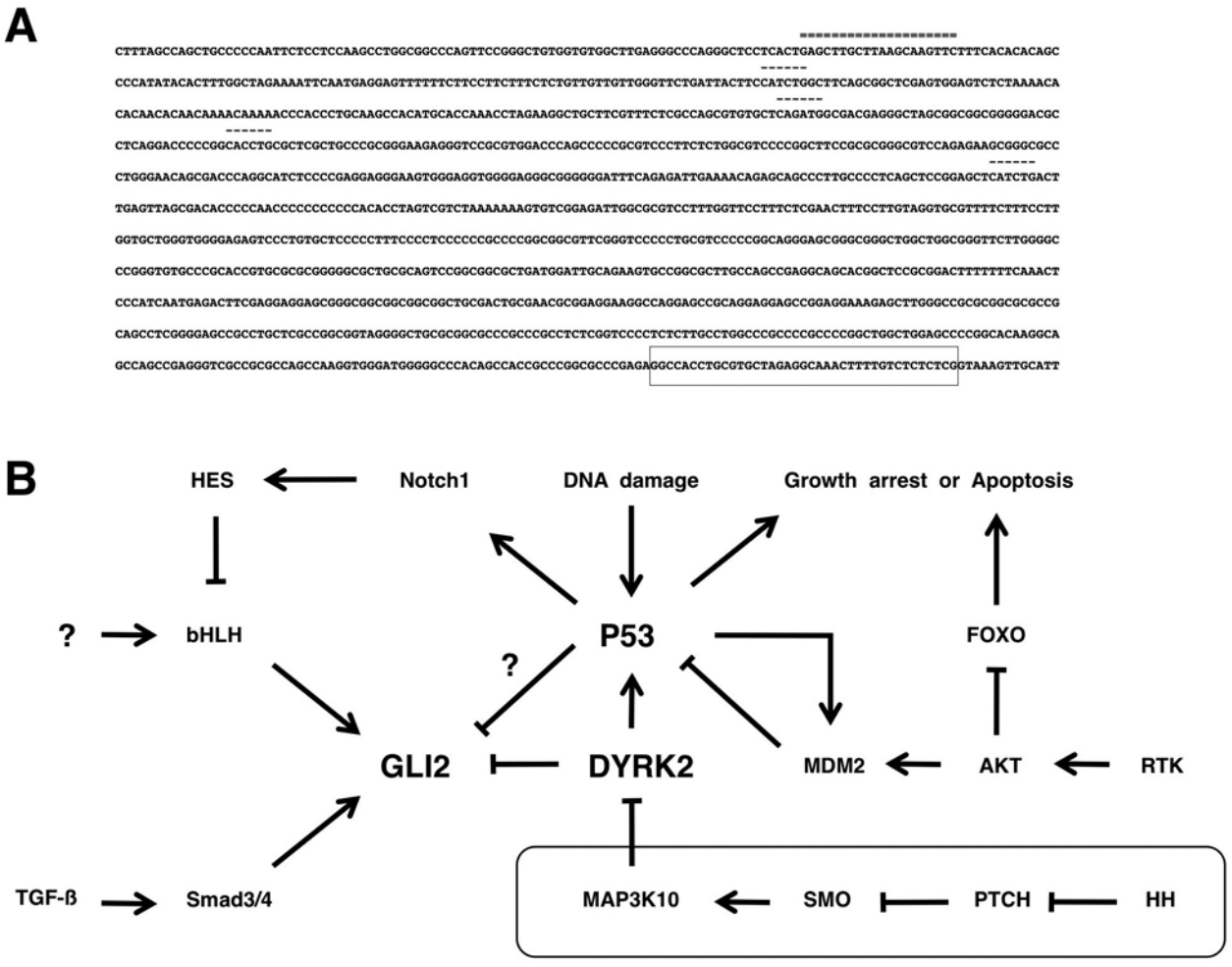

Figure 2. GLI2 and P53. (A), Proximal promoter region of human GLI2. Tandem P53-binding sites (double overline), conserved bHLH-binding sites (overline), and exonic region (open box) are indicated. (B), Interaction map of P53 and stem cell signaling network. DYRK2 is located at the crossroads of Hedgehog and P53 signaling cascades.

We identified tandem P53-binding motifs within the human *GLI2* promoter (Fig. 2A). *GLI2* was not listed on the 542 target genes or loci of P53 identified by using ChIP-on-PET approach (42), nor on the 1546 targets of P53 identified by using ChIP-on-chip approach (49). P53, P63, and P73 bind to the tandem RRRCWWGYYY motifs to activate or repress their target genes. Although transcriptional regulation of *GLI2* by P53, P63, and P73 should be further investigated, this is the first report on the tandem P53-binding motifs within the proximal promoter region of human *GLI2* gene.

DYRK2 phosphorylates P53 to enhance apoptotic response to DNA damage (50), while DYRK2 phosphorylates GLI2 and NFATc to prime subsequent phosphorylation by GSK3ß for degradation and inhibition of nuclear translocation, respectively (23,51). Hedgehog signaling activation leads to DYRK2 inhibition in the cytoplasm, which might lead to inhibition of DYRK2-mediated P53 activity in the nucleus. Because DYRK2, HIPK2, PKCδ and P38 are implicated in P53 activation (50), Hedgehog signals are predicted to partially inhibit P53 activity in cancer cells.

It was expected that *DYRK2* gene might be deleted, mutated or silenced in some cancer as a tumor suppressor gene; however, *DYRK2* gene is amplified and overexpressed in lung cancer, esophageal cancer, and gastric cancer (52,53). The mechanism how DYRK2 overexpression leads to survival or proliferation of tumor cells remains to be elucidated.

MDM2 and MDM4 (MDMX) are negative regulators of P53. MDM2 and MDM4 share the common domain architecture consisting of P53-binding domain, acidic domain, zinc finger domain, and RING finger domain (54-57). *MDM2* gene, located at human chromosome 12q15, is amplified and overexpressed in osteosarcoma, soft tissues sarcoma, gastric cancer, lung cancer, and esophageal cancer (52,55,58). *MDM4* gene, located at human chromosome 1q32.1, is amplified and overexpressed in malignant glioma, breast cancer, and retinoblastoma (59-61). *MDM2* and *MDM4* function as oncogenes, because MDM2 and MDM4 proteins protect tumor cells from apopotosis or growth arrest due to their inhibitory effect on P53 tumor suppressor.

Receptor tyrosine kinase (RTK)-mediated or RTK-induced PI3K-AKT signaling activation leads MDM2 phosphorylation at Ser 166 and Ser 186 to inhibit P53 activity (62). Because the activation of Hedgehog signaling and RTK signaling synergistically inhibits P53 function (Fig. 2B), monitoring of P53, Hedgehog, and AKT activities in primary tumors could be utilized for the prediction of effectiveness of DNA-damaging agent or irradiation to induce P53-mediated apoptosis in cancer cells. In addition, combination chemotherapy using

Hedgehog inhibitor and RTK inhibitor could promote anti-tumor effects of chemotherapy and/or irradiation.

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