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SHP (small heterodimer partner) suppresses the transcriptional activity and nuclear localization of Hedgehog signalling protein Gli1

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Gli (glioma-associated oncogene homologue) proteins act as terminal effectors of the Hedgehog signalling pathway, which is implicated in the development of many human malignancies. Gli activation is important for cell proliferation and anti-apoptosis in various cancers. Several studies have suggested that nuclear receptors have anti-cancer effects by inhibiting the activation of various oncoproteins. However, the involvement of nuclear receptors on the Hedgehog/Gli signalling pathway is poorly defined. In the present study we identified SHP (small heterodimer partner) as a nuclear receptor that decreased the expression of Gli target genes by repressing the transcriptional activity of Gli1. The inhibitory effect of SHP was associated with the inhibition of Gli1 nuclear localization via protein–protein interaction. Finally, SHP overexpression decreased the expression of Gli target genes and SHP knockdown increased the expression of these genes. Taken together, these results suggest that SHP can play a negative role in Hedgehog/Gli1 signalling.

Key words: cancer, glioma-associated oncogene homologue 1 (Gli1), Hedgehog, small heterodimer partner (SHP), tumorigenesis.

INTRODUCTION

Gli (glioma-associated oncogene homologue) was identified as a gene that is amplified in gliomas [1] and it acts as a terminal effector of the Hedgehog signalling pathway [2]. Typically, Hedgehog signalling is initiated by the binding of Hedgehog ligand to the Ptch (patched) receptor. The binding of ligand to receptor is able to alleviate the Ptch-mediated suppression of Smo (smoothened). Activated Smo subsequently increases Gli activation by inducing nuclear translocation, leading to the increased expression of Gli target genes [3]. Mutations in various components and inappropriate activation of the Hedgehog signalling pathway are implicated in several human malignancies (e.g. medulloblastoma, rhabdomyosarcoma, and skin, breast, lung, stomach, pancreas, liver, ovary, prostate, bladder, colon, biliary and oesophagus cancer) [4–14]. Three Gli family transcriptional factors (Gli1, Gli2 and Gli3), which share a highly conserved zinc-finger domain, have been identified in mammals. Gli1 and Gli2 are transcriptional activators of Hedgehog target genes, whereas Gli3 acts mainly as a repressor. Numerous studies have reported that the activated transcriptional functions of Gli1 or Gli2 are important for cell proliferation and anti-apoptosis in a variety of human cancers [12,15–18]. Furthermore, earlier studies in frogs and mice have shown that Gli1 or Gli2 overexpression induces skin tumours [19–21]. Taken together, these findings indicate that Gli plays a significant role in Hedgehog signalling during tumorigenesis.

SHP (small heterodimer partner) is an atypical member of nuclear receptor superfamily consisting of a putative ligand-binding domain, but lacking a conventional DNA-binding domain [22]. SHP interacts with conventional nuclear receptors and negatively regulates their transcriptional activities [23]. Studies with SHP-knockout or -transgenic mice have identified a broader role for SHP in the regulation of energy balance in brown fat, glucose homoeostasis and hepatic lipid metabolism [24–26]. Furthermore, SHP protects against hepatic fibrosis by inhibiting hepatic stellate cells [27]. Interestingly, recent studies have suggested that SHP may have a tumour-suppressive function in hepatocellular carcinoma [28,29]. However, the molecular mechanism by which SHP regulates tumorigenesis remains unknown.

Despite the importance of Gli as oncogene in tumorigenesis, the negative regulators that inhibit the transcriptional activity of Gli are not well defined. It has been reported that several nuclear receptors, such as SHP, LXR (liver X receptor) and PPAR (peroxisome-proliferator-activated receptor), have potent anti-cancer effects [30–33]. Previous reports have also demonstrated the negative regulatory effects of LXR on Hedgehog signalling in pluripotent mesenchymal cells (marrow stromal cells) and embryonic fibroblasts [34]. Therefore we investigated whether these nuclear receptors suppress the transcriptional activity of Gli1 in cancer cells. We show that SHP inhibits the transcriptional activity and nuclear translocation of Gli1 via protein–protein interactions. We also found that SHP overexpression decreases the expression of Gli target genes, whereas SHP-knockdown increases the expression of these genes. These findings provide the first evidence that SHP acts as an inhibitory regulator in the Hedgehog/Gli signalling pathway.

EXPERIMENTAL

Plasmid constructs and reagents

pCMX-hLXRα was a gift from Dr David J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas,

Abbreviations used: AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid; ChIP, chromatin immunoprecipitation; CRM1, chromosome region maintenance homologue 1; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; Gli, glioma-associated oncogene homologue; GST, glutathione transferase; HA, haemagglutinin; HDAC, histone deacetylase; HEK, human embryonic kidney; HNF4α, hepatocyte nuclear factor 4α; LMB, leptomycin B; LXR, liver X receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NES, nuclear export sequence; NP-40; Nonidet P40; PPAR, peroxisome-proliferator-activated receptor; Ptch, patched; qRT-PCR, quantitative real-time PCR; RFP, red fluorescent protein; RT, reverse transcription; SAG, small-molecule activator of smoothened; SHP, small heterodimer partner; shRNA, small-hairpin RNA; Smo, smoothened; Sufu, suppressor of fused homologue; TSA, trichostatin A.

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TX, U.S.A.). pCMV-Myc-hSuFu and pCMV5-Smo-HA were provided by Dr Rune Toftgard (Center for Nutrition and Toxicology, Karolinska Institute, Stockholm, Sweden). pcDNA3.1-HA-G9a-WT and pcDNA3.1-HA-G9a-DN were a gift from Dr Kenneth L. Wright (University of South Florida, Tampa, FL, U.S.A.). The 8×Gli-bs-luc reporter (containing a promoter consisting of eight Gli-binding sites fused to the luciferase reporter) and pcDNA3.1-HisB-Gli3 were provided by Dr Hiroshi Sasaki (RIKEN Center for Developmental Biology, Kobe, Japan). The *PTCH1*-luc reporter and *BCL-2*-luc reporter contructs (containing the *PTCH1* and *BCL-2* promoters fused to the luciferase reporter respectively) were a gift from Dr Fritz Aberger (University of Salzburg, Salzburg, Austria). pcDNA3-FLAG-K-Ras-(G12C) was kindly provided by Dr Bum-Joon Park (Pusan National University, Busan, Republic of Korea). pcDNA3-HA-hSHP, pGL2B-hSHP-luc and pGL2B-mSHP-luc [containing the 2.2 kb of the *hSHP* (human SHP) and *mSHP* (mouseSHP) promoter fused to the luciferase reporter respectively] were a gift from Dr Heung Sik Choi (Chonnam National University, Gwangju, Republic of Korea). The Gal4-tk-luc reporter vector was provided by Dr Ronald M. Evans (Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, CA, U.S.A.). pcDNA3-HA-hGli1 and pcDNA3-GFP-hGli1 were constructed by inserting the fragments of Gli1 [provided by Dr Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD, U.S.A.)] into the pcDNA3-HA and pcDNA3-GFP vector. To create pcDNA3-GFP-Gli1/NESmut, two leucine residues of pcDNA3-GFP-Gli1 were mutated to alanine (Leu^{501} and Leu^{503}) by DpnI-based site-directed mutagenesis. To create pCS2-GFP-hGli2, GFP (green fluorescent protein) fragments were inserted into HindIII/EcoRI-digested pCS2MT-hGli2 [a gift from Dr Maximilian Muenke (National Human Genome Research Institute, Bethesda, MD, U.S.A.)]. pcDNA3-6Myc-hGli2 was cloned by inserting HindIII/XbaI-digested Myc_6–hGli2 into HindIII/XbaI-digested pcDNA3 (Invitrogen). To obtain pcDNA3-RFP [for C-terminal RFP (red fluorescent protein) tagging], the RFP sequence was inserted into the multi-cloning site of pcDNA3. pcDNA3-HA-hSHP-RFP was constructed by inserting the SHP fragments fused to the HA (haemagglutinin) coding sequence into pcDNA3-RFP. To make pcDNA3-GST-hSHP, SHP fragments were inserted into EcoRI/XhoI-digested pcDNA3-GST. To create the pM-rHNF4α vector, rHNF4α [rat HNF (hepatocyte nuclear factor) 4α] fragments (provided by Dr Heung Sik Choi) were inserted into the pM vector (Clontech). pSPORT-mPPARγ2 and pcDNA3-hPPARα have been described previously [35]. The identity of all of the plasmids was confirmed by automatic sequencing analysis. AHPN {6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid}, an SHP agonist, and LMB (leptomycin B) were purchased from Sigma–Aldrich. Rosiglitazone, a PPARγ agonist, and TO901317, an LXR agonist, were obtained from Cayman Chemical. The transfection reagents Polyfect and jetPEI were purchased from Qiagen and Polyplus Transfection respectively. SAG (small-molecule activator of Smo) was from Calbiochem and TSA (trichostatin A) was from Sigma.

Cell culture

SKBR3, HEK (human embryonic kidney)-293 and HeLa cells were obtained from the A.T.C.C. Huh7 cells were a gift from Dr Hyeseong Cho (Ajou University, Suwon, Republic of Korea). All cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS (fetal bovine serum) at 37 °C in a humid atmosphere of 5 % CO_2.

Co-immunoprecipitation, *in vivo* GST (glutathione transferase) pull-down, nuclear/cytosolic fraction preparation and Western blotting

Cells were lysed in a radio-immunoprecipitation assay buffer [RIPA; 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) NP-40 (Nonidet P40), 0.25 % sodium deoxycholate, 1 mM PMSF and protease inhibitors (Roche)] for 30 min on ice, and whole-cell lysates were obtained by subsequent centrifugation at 13 000 ***g*** for 20 min at 4 °C. Cell lysates (50 μg of protein) were subjected to SDS/PAGE (6–18 % gels) and transferred on to a PVDF membrane (Millipore) by semi-dry electroblotting. The membranes were then incubated with an anti-actin antibody [A2066 or sc-1616 (Sigma–Aldrich and Santa Cruz Biotechnology respectively)], an anti-GST antibody (sc-138; Santa Cruz Biotechnology), an anti-HA antibody (1 867 423; Roche), an anti-(Lamin A) antibody (sc-20680; Santa Cruz Biotechnology), an anti-GFP antibody (sc-9996; Santa Cruz Biotechnology) or an anti-Myc antibody (sc-789; Santa Cruz Biotechnology) in TBST (Tris-buffered saline containing 1 % Tween-20) supplemented with 1 % (w/v) non-fat dry milk. The bands were detected using an ECL (enhanced chemiluminescence system) (Amersham Biosciences).

For co-immunoprecipitation, cell extracts were mixed with the anti-HA antibody or anti-SHP (sc-15283; Santa Cruz Biotechnology) antibody immobilized on Protein G–Sepharose (Invitrogen) and incubated for 2 h in a cold-room. After washing with RIPA buffer, immunoprecipitates were collected by centrifugation at 13 000 ***g*** for 2 min at 4 °C and proteins were dissolved in Laemmli buffer. For the *in vivo* GST pull-down assay, cell extracts expressing HA–Gli1 or GFP–Gli1 and GST–SHP fusion proteins were incubated with glutathione–Sepharose beads (Amersham Biosciences) for 2 h in a cold-room. After washing with ice-cold PBS, the samples were dissolved in Laemmli buffer and boiled. After centrifugation at 13 000 ***g*** for 2 min at 4 °C, the supernatants were subjected to SDS/PAGE (6–12 % gels) and transferred on to PVDF membranes.

To prepare cytosolic and nuclear lysates, cells were lysed in buffer A [10 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT (dithiothreitol), 0.5 % NP-40, 1mM PMSF and protease inhibitors] and incubated for 10 min on ice. The supernatants (cytosolic lysates) were collected by centrifugation at 3300 ***g*** for 5 min at 4 °C. The nuclear pellets were then washed with ice-cold PBS to avoid contamination of cytosolic proteins and lysed in buffer B [10 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 25 % (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 % NP-40, 1 mM PMSF and protease inhibitors]. After incubation on ice for 25 min, the supernatants (nuclear lysates) were collected by centrifugation at 13 400 ***g*** for 5 min at 4 °C.

Luciferase assays

Cells were seeded in a 24-well culture plate and transfected with 0.1 μg of both the reporter vector and the β-galactosidase expression plasmid, along with each indicated expression plasmid, using PolyFect. After 24–48 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

RNAi (RNA interference)

The knockdown of human SHP was performed by using the pSUPER vector system. pSUPER-hSHP was provided by Dr Jongsook Kim Kemper (University of Illinois, Champaign, IL, U.S.A.). pSUPER-GFP was constructed using a 19-nucleotide sequence of the GFP-coding sequences and used as control vector. Cells were transfected with the shRNA (small-hairpin RNA) constructs using jetPEI according to the manufacturer's instructions.

RNA isolation, RT (reverse transcription)–PCR and qRT-PCR (quantitative real-time PCR)

Total RNA from various transfected cells was prepared using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was converted into single-strand cDNA with the MMLV (Moloney-murine-leukaemia virus) RT (Promega) with random hexamer primers. An aliquot (1/20 vol.) of the cDNA was subjected to PCR amplification using gene-specific primers. The following PCR primers were used: Ptch1, 5′-TTCTCACAACCCTCGGAACCC A-3′ (forward) and 5′-CTGCAGCTCAATGACTTCCACCTT-3′ (reverse); Gli1, 5′-CACACAAGTGCACGTTTGAAGGGT-3′ (forward) and 5′-ACTGTAGAAATGGATGGTGCCCGA-3′ (reverse); Gli2, 5′-TGGCCTACATCAACAACTCCCGAA-3′ (forward) and 5′-CTTGACCTTGCTGCGCTTGTGAAT-3′ (reverse); Bcl-2, 5′-GTTCGGTGGGGTCATGTGTGTGGAGAGCG-3′ (forward) and 5′-TAGCTGATTCGACGTTTTGCCTGA-3′ (reverse); Bcl-X_L, 5′-AAAATGTCTCAGAGCAACCGGGAGCTG-3′ (forward) and 5′-TCATTTCCGACTGAAGAGTGAGCCCAG-3′ (reverse); SHP, 5′-AGCTATGTGCACCTCATCGCACCTGC-3′ (forward) and 5′-CAAGCAGGCTGGTCGGAATGGACTTG-3′ (reverse); β-actin, 5′-GACTACCTCATGAAGATC-3′ (forward) and 5′-GATCCACATCTGCTGGAA-3′ (reverse); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GTGGTCTCCTCTGACTTCAAC-3′ (forward) and 5′-TCTCTTCCTCTTGTGCTCTTG-3′ (reverse). The RT–PCR bands were quantified and normalized relative to the β-actin mRNA control band with ImageJ version 1.40 (NIH). qRT-PCR was performed with an SYBR Green I LightCycler-based real-time PCR assay (Roche Applied Science). The reaction mixtures were prepared using LightCycler Fast Start DNA master mixture for SYBR Green I, 0.5 μM of each primer and 4 mM $MgCl_2$. All PCR conditions and primers were optimized to produce a single product of the correct base pair size.

Fluorescence microscopy in living cells

Fluorescence microscopy was performed on SKBR3 cells transfected with the pcDNA3-GFP-hGli1 expression construct, along with the indicated expression plasmid, using jetPEI. Following transfection, cells were incubated for 24 h. Prior to imaging, cells were counterstained with Hoechst dye for 10 min at 37 °C to stain the nuclei. Cells were visualized with a Zeiss Axiovert 200M microscope.

ChIP (chromatin immunoprecipitation) assays

Cells were seeded and transfected with the indicated plasmid using jetPEI. After 36 h of transfection, cells were washed twice with PBS and cross-linked with 1 % (w/v) formaldehyde for 10 min at 37 °C. Glycine (125 mM) was added for 5 min at room temperature (24 °C) to stop the reaction. Chromatin solutions were sonicated using a Sonics VC130 instrument at power output setting 5 seven times with 10 s intervals and incubated with the anti-GFP or anti-His antibodies, or control IgG, overnight at 4 °C with rotation. The immune complexes were collected with Protein G–Sepharose slurry (Invitrogen) and salmon-sperm DNA for 4 h with rotational washing and then incubated overnight at 65 °C to reverse the cross-linking. Purified DNA was subjected to PCR using primers flanking the Gli-binding motif in the human *PTCH1* promoter. The following PCR primers were used: 5′-CCTTAATGGAAGTATTGCATGCG-3′ (forward) and 5′-CTGTCAGATGGCTTGGGTTTCTG-3′ (reverse). The PCR products were 203-bp in length.

Cell viability assay

Cell viability was determined via an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. In brief, Huh7 cells transfected with the Gli-bs-luc reporter were seeded in 24-well culture plates and treated with AHPN at the indicated concentrations for 24 h. The medium was removed and replaced with complete cell culture medium containing 0.5 mg/ml MTT (Sigma–Aldrich) for 1 h at 37 °C. After discarding the medium, DMSO was added. The cell suspensions were then removed and placed into 96-well trays for analysis. Absorbance values were determined at 570 nm.

Statistical analysis

Statistical analyses were carried out using unpaired or paired Student's *t* tests as appropriate. All results are reported as means $\pm$ S.D., and a P value of <0.05 was considered significant.

RESULTS

SHP suppresses the transcriptional activity of Gli1 in various cancer cell lines

To investigate whether nuclear receptors with anti-cancer activity affect Gli1 signalling, we examined the transcriptional activity of Gli1 by nuclear receptors using a Gli-binding site luciferase reporter vector (Gli-bs-luc). As shown in Figure 1(A), the transcriptional activity of Gli1 was inhibited by SHP, but not by LXRα or PPARγ, in various cancer cell lines, such as Huh7 (Figure 1A, left-hand panel), HeLa (Figure 1A, right-hand panel) and SKBR3 (results not shown) cells. We also found that LXRβ, PPARα or PPARβ/δ overexpression had no effect on the activation of *Gli1* transcription (results not shown). The SHP-mediated inhibitory effect on Gli1 activity was confirmed by treatment with AHPN, which acts as an agonist of SHP. However, the agonists of other nuclear receptors did not affect the Gli1 transcriptional activity (Figure 1B). In addition, AHPN treatments did not affect cell viability as measured by an MTT assay (Figure 1C, left-hand panel); however, addition of AHPN significantly enhanced the SHP-mediated repression of Gli1 activity (Figure 1C, right-hand panel). We also examined the effect on Hedgehog/Gli target genes in the absence or presence of AHPN and/or the Shh (sonic Hedgehog) agonist SAG. As shown in Figure 1(D), treatment with SAG significantly increased the mRNA levels of the Hedgehog/Gli target genes *PTCH1*, *Gli1*, *Bcl-2* and *Bcl-X_L*. In addition, although *Gli2* has been not reported as a target gene of the Hedgehog/Gli signalling pathway, we showed that SAG increased *Gli2* mRNA expression. The mRNA level of these genes was decreased by treatments with AHPN, even in the presence of SAG (Figure 1D). Moreover, SHP suppressed the Smo-induced increase in Gli1 transcriptional activity (Figure 1E). We also found that SHP inhibited the transcriptional activity of Gli2 (see Supplementary Figures S1A–S1C at http://www.BiochemJ.org/bj/427/bj4270413add.htm) in

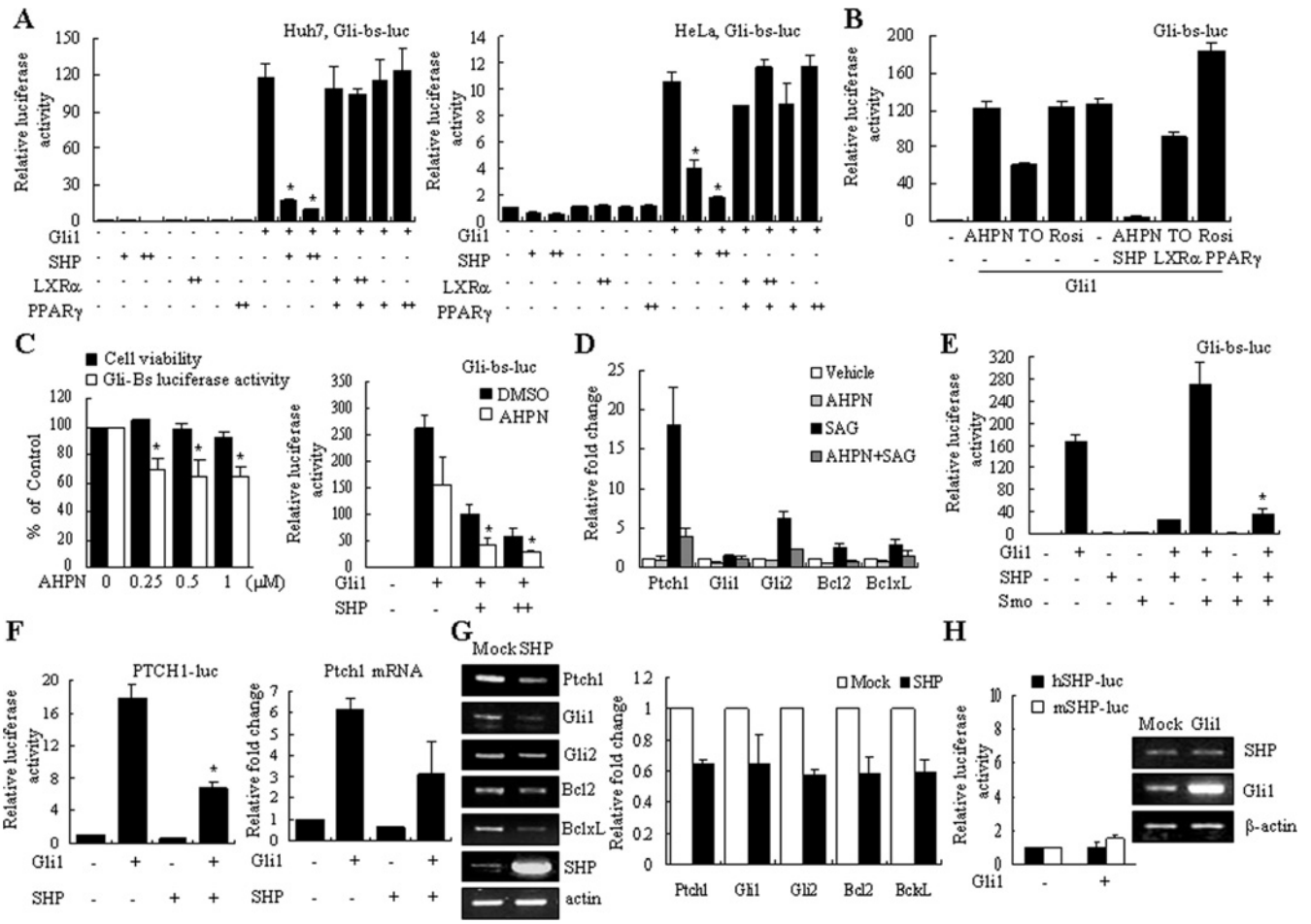

Figure 1 Effect of SHP on Gli1 transcriptional activity and Gli target gene expression

(**A**) Identification of SHP as a negative regulator on Gli1 transcriptional activity. Huh7 (left-hand panel) and HeLa (right-hand panel) cells were transfected with 0.1 μg of Gli1 and 0.1 (+) or 0.2 μg (++) of the indicated nuclear receptor expression plasmids, along with 0.1 μg of the Gli-bs-luc reporter plasmid. After 24 h of transfection, cell lysates were obtained and luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). *$P < 0.05$ compared with Gli1 transfectants. (**B**) The effect of agonists of various nuclear receptors on Gli1 transcriptional activity. Huh7 cells were transfected with 0.1 μg of Gli1 and 0.2 μg of the indicated nuclear receptor expression plasmids, along with 0.1 μg of the Gli-bs-luc reporter plasmid. After 24 h of transfection, cells were treated with 0.5 μM AHPN, 10 μM TO901317 (TO), 10 μM rosiglitazone (Rosi) or vehicle (−) for 24 h. Cells were harvested and luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). *$P < 0.05$ compared with vehicle-treated Gli1 transfectants. (**C**) The effect of AHPN on cell viability (left-hand panel). Huh7 cells were transfected with 0.1 μg of Gli-bs-luc reporter plasmid. After 24 h of transfection, cells were treated with AHPN at the indicated concentrations for 24 h. Cell viability was measured with the MTT assay. Results are means $\pm$ S.D. ($n = 3$). *$P < 0.05$ compared with vehicle-treated transfectants. The effect of AHPN on SHP-mediated repression of Gli1 activity (right-hand panel). Huh7 cells were transfected with the indicated plasmids [0.1 (+) or 0.2 μg (++)] and the Gli-bs-luc reporter plasmid. After 24 h of transfection, cells were treated with 0.5 μM AHPN for 24 h. Cells were harvested and luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). *$P < 0.05$ compared with vehicle-treated transfectants. (**D**) The effect of SAG and/or AHPN on Hedgehog/Gli target gene expression. HeLa cells were incubated with 1 μM SAG and/or 1 μM AHPN for 48 h in serum-free medium. The mRNA levels of the indicated genes were determined using qRT-PCR. (**E**) SHP represses Smo-induced Gli1 transcriptional activity. Huh7 cells were transfected with 0.1 μg of Gli1 and 0.4 μg of Smo expression plasmids as indicated, along with 0.1 μg of the Gli-bs-luc reporter plasmid. After 24 h of transfection, cell lysates were obtained and luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). Note that the transcriptional activity of Gli1 was mildly but significantly increased by transfection of Smo (*$P < 0.05$ compared with Smo-expressing Gli1 transfectants). (**F**) The effect of SHP on Gli1-mediated *PTCH1* gene expression. After 24 h of transfection (Huh7 cells), *PTCH1*–luciferase activity (left-hand panel) was assessed and qRT-PCR (right-hand panel) was performed to measure the *PTCH1* mRNA levels. Results for the luciferase assay are means $\pm$ S.D. ($n = 3$). *$P < 0.05$ compared with Gli1 transfectants. (**G**) The effect of SHP on Hedgehog/Gli target gene expression. HeLa cells were transfected with 2 μg of SHP expression plasmid and empty vector (Mock). After 36 h of transfection, RT–PCR (left-hand panel) or qRT-PCR (right-hand panel) was performed. The qRT-PCR data were normalized relative to the *GAPDH* mRNA level. (**H**) Gli1 has no effect on *SHP* gene expression. Huh7 cells were transfected with indicated plasmids. Luciferase activity was assessed (left-hand panel) and RT–PCR was performed (right-hand panel). Results for the luciferase assay are means $\pm$ S.D. ($n = 3$).

Huh7, SKBR3 and HeLa cells. Next, to check whether SHP could repress the expression of the Gli1 target genes, we performed a luciferase assay with a reporter containing the promoter of *PTCH1*, which is used as marker of Hedgehog/Gli signalling activation [36]. Luciferase activity results revealed that SHP reduced the Gli1-mediated increase in *PTCH1*–luciferase activity (Figure 1F, left-hand panel). These inhibitory effects of SHP were confirmed by analysing the mRNA level of *PTCH1* using qRT-PCR (Figure 1F, right-hand panel). To further investigate the changes in mRNA level of the Gli target genes in the presence of SHP, RT–PCR and qRT-PCR analysis was performed in cells overexpressing SHP. As shown in Figure 1(G), SHP decreased the expression of *PTCH1*, *Gli1*, *Gli2*, *Bcl-2* and *Bcl-X*$_L$. However, in contrast with the effect of SHP on *Gli* gene expression, Gli had no effect on *SHP* gene expression (Figure 1H and Supplementary Figure S1E). Taken together, these results suggest that SHP inhibits the expression of Gli target genes by repressing the transcriptional activity of Gli1.

SHP interacts with Gli1 protein

To determine whether the repression of the Gli1 transcriptional activity by SHP is mediated by Gli1–SHP interaction, we

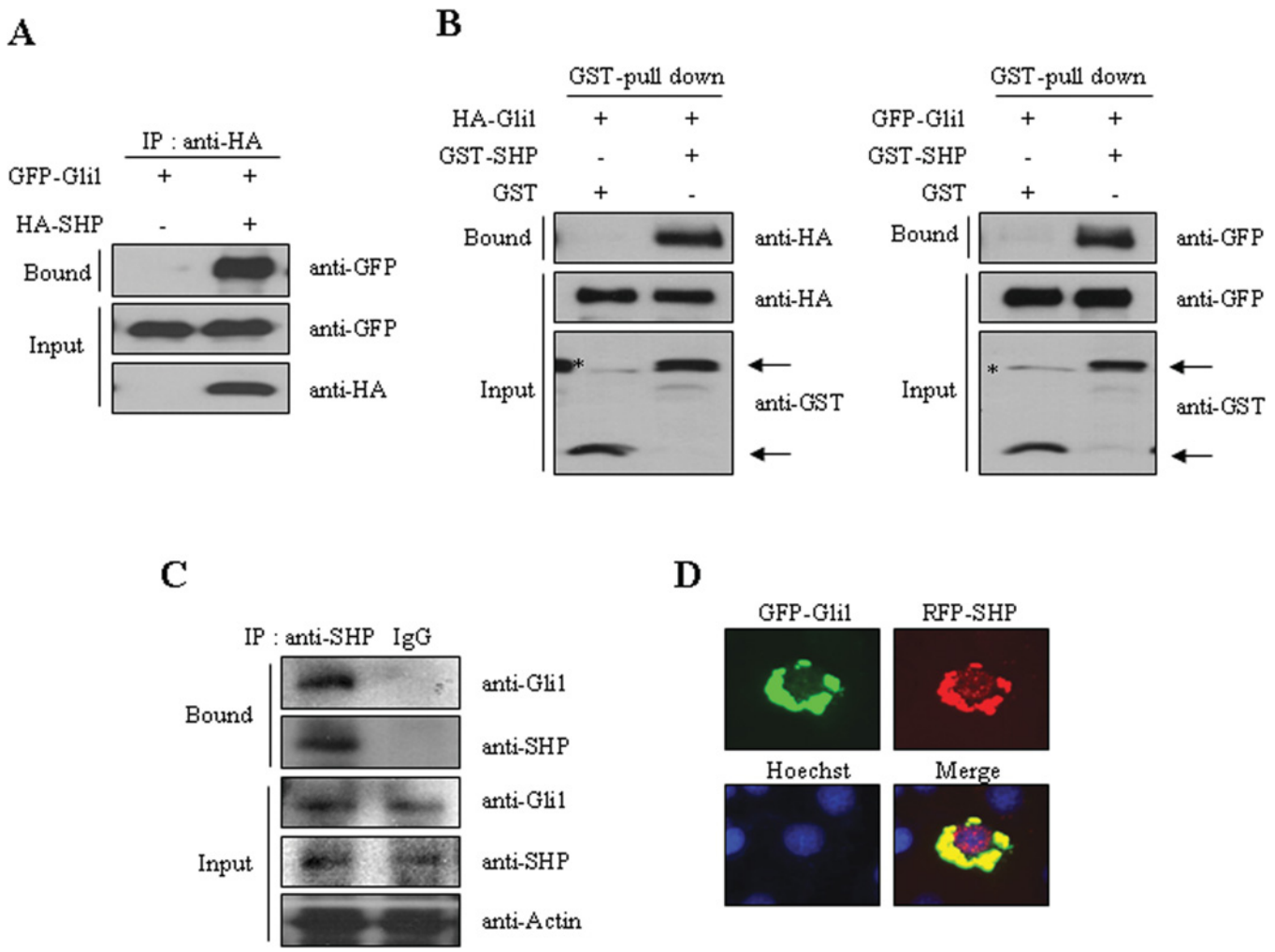

Figure 2 SHP interacts with Gli1

(**A**) The interaction between HA–SHP and GFP–Gli1 by co-immunoprecipitation assay. HEK-293 cells were transfected with HA–SHP or empty vector, as indicated, along with GFP–Gli1. After 36 h of transfection, cell lysates were obtained and immunoprecipitated (IP) with anti-HA antibody, followed by Western blotting using anti-GFP antibody. A representative blot is shown from three independent experiments. (**B**) The interaction between GST–SHP and HA–Gli1/GFP–Gli1 by *in vivo* GST pull-down assay. HEK-293 cells were transfected with vectors expressing GST alone or GST–SHP proteins, along with HA–Gli1 or GFP–Gli1 expression vectors. After 36 h of transfection, cell lysates were obtained and immobilized on to glutathione–Sepharose beads, followed by Western blotting using the indicated antibodies. The asterisk indicates a non-specific band in each blot, and the arrows point to specific bands. (**C**) The interaction between endogenous Gli1 and SHP. HeLa cells extracts were co-immunoprecipitated (IP) using anti-SHP antibodies and assessed by immunoblotting using the indicated antibodies. There was an interaction between SHP and Gli1 protein. IgG served as a negative control. (**D**) Co-localization of SHP and Gli1 in the cytoplasm and nucleus. Both GFP–Gli1 and RFP–SHP plasmids were co-transfected into SKBR3 cells. After 24 h of transfection, cells were counterstained with Hoechst to label nuclei and cell imaging was assessed by fluorescence microscopy. Images shown are representative of three independent experiments.

performed a co-immunoprecipitation assay. In HEK-293 cells co-transfected with HA–SHP and GFP–Gli1 fusion constructs, HA–SHP protein was co-immunoprecipitated with GFP–Gli1 (Figure 2A). To further confirm a physical interaction between SHP and Gli1, an *in vivo* GST pull-down assay was performed. We observed that Gli1 bound to GST–SHP, but not GST protein (Figure 2B). Furthermore, we examined the interaction between SHP and Gli1 on the endogenous levels of the proteins using an anti-SHP antibody (Figure 2C). Both the co-immunoprecipitation and the *in vivo* GST pull-down assay results suggest that SHP may co-localize with Gli1. To investigate this possibility, cells were co-transfected with GFP–Gli1 and RFP–SHP expression plasmids, and the subcellular localization was then examined by fluorescence microscopy in living cells. As shown in Figure 2(D), SHP co-localized with Gli1 in both the cytosol and nuclei. In addition, SHP interacted with Gli2 through co-localization (Supplementary Figure S1D). Thus these results indicate that SHP physically interacts with Gli protein.

SHP partially suppresses the nuclear localization of Gli1

We next analysed the mechanism by which SHP inhibits the transcriptional activity of Gli1. It has been reported that SHP can repress the transcriptional activity via recruitment of the histone methyltransferase G9a [37,38]. We therefore investigated whether SHP-mediated inhibition of Gli1 transcriptional activity also involved G9a. As shown in the left-hand panel of Figure 3(A), wild-type G9a had no effect on the SHP-inhibited transcriptional activity of Gli1. We also showed that dominant-negative G9a (G9a with a 'dead' methyltransferase activity; a mutant G9a where Asn^{903} and His^{904} are replaced by leucine and glutamic acid residues respectively [37]) had no effect. In addition, it was shown previously that HDACs (histone deacetylases) play a role

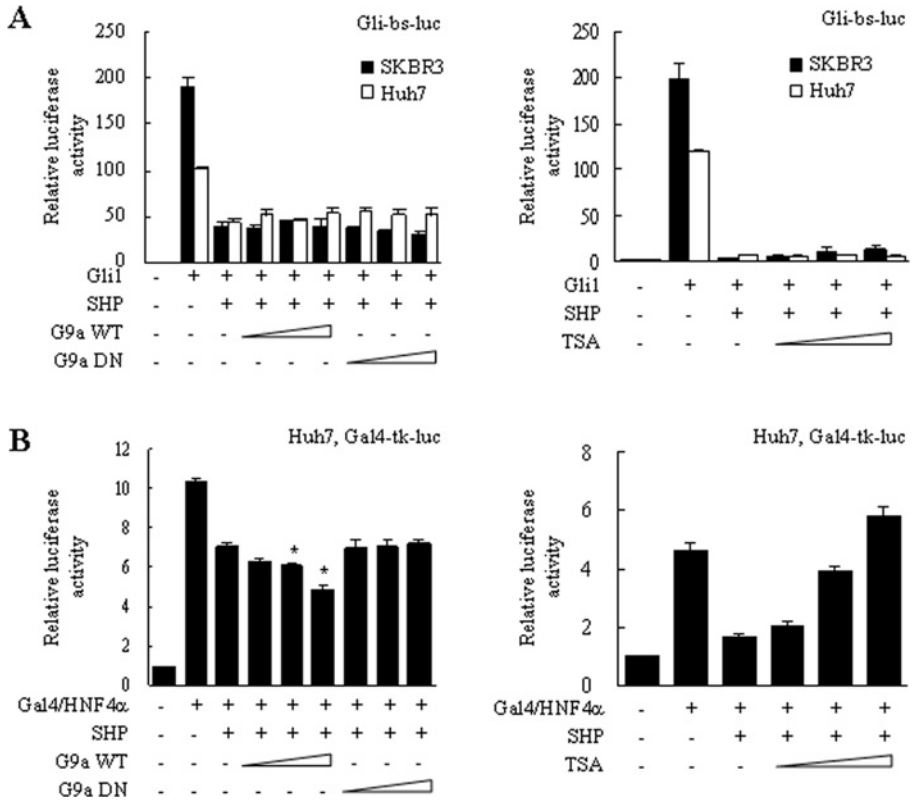

Figure 3 Effects of G9a and HDAC inhibitor on SHP-repressed Gli1 transcriptional activity

(**A**) G9a has no effect on the SHP-mediated inhibition of Gli1 activity (left-hand panel). Cells were transfected with 0.1 μg of Gli1, 0.025 μg of SHP, and either wild-type (WT) or dominant-negative (DN) G9a expression plasmids (0.1, 0.2 or 0.4 μg), along with 0.1 μg of the Gli-bs-luc reporter plasmid. After 24 h of transfection, cell lysates were obtained and luciferase activity was measured. TSA had no effect on SHP-mediated suppression of Gli1 activity (right-hand panel). Cells were transfected with Gli1 (0.1 μg) and SHP (0.2 μg) expression plasmids, along with 0.1 μg of Gli-bs-luc reporter plasmid. After 24 h of transfection, cells were treated with TSA (50, 100 and 200 nM concentrations) for 24 h. Cells were harvested and luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). (**B**) The effects of G9a and TSA on SHP-mediated inhibition of HNF4α activity. Huh7 cells were transfected with the indicated plasmids and where indicated treated with TSA (50, 100 and 200 nM concentrations) for 24 h. Luciferase activity was measured. Results are means $\pm$ S.D. ($n = 3$). $^*P < 0.05$ compared with HNF4α transfectants not expressing SHP.

in SHP-mediated repression [37,39]. To test whether HDACs mediate the SHP repression on Gli1 activity, we exploited an HDAC inhibitor, TSA. As shown in the right panel of Figure 3(A), TSA treatment did not attenuate the SHP-mediated inhibition of Gli1 activity. Taken together, these results suggest that the inhibitory effect of SHP on Gli1 transcriptional activity is not associated with the recruitment of histone modification enzymes (G9a and HDACs).

As Gli1 is a nuclear cytoplasmic shuttling protein [40], we attempted to determine whether SHP affects the subcellular localization of Gli1. We found that SHP transfection partially resulted in an increase in the proportion of cells with cytosolic Gli1 and a decrease in both the proportion with nuclear Gli1 and, nuclear and cytosolic Gli1 (Figures 4A and 4B). As nuclear export of Gli1 is CRM1 (chromosome region maintenance homologue 1)-dependent [40], we examined Gli1 localization in the presence of LMB, a CRM1-dependent export inhibitor. We found that LMB treatment induced an increase in Gli1 nuclear localization, which was partially attenuated by SHP (Figure 4C). To confirm these effects, we generated Gli1-NESmut, in which two amino acids (Leu^{501} and Leu^{503}) in the NES (nuclear export sequence) were replaced with alanine residues. As shown in the Figure 4(C), SHP repressed the nuclear localization of Gli1-NESmut protein. In addition, we tested whether SHP inhibited the ability of Gli1 to bind to DNA of the promoter region of the target gene in cells. As expected, owing to the inhibition of Gli1 nuclear localization, SHP suppressed the level of Gli1 binding to the promoter region of the *PTCH1* gene (Figure 4D). These findings suggest that SHP partially represses the nuclear localization of Gli1.

SHP inhibits the Gli1 activity in co-operation with Sufu (suppressor of fused homologue), but not Gli3

It was possible that the suppression of transcriptional activity of Gli1 by SHP resulted from the induction of negative regulators of Hedgehog/Gli signalling. To determine whether this was the case, we examined the effect of negative regulators of Hedgehog/Gli signalling in SHP-mediated repression of Gli1 activity. First, we tested the effect of Gli3, one of the repressors of Hedgehog/Gli signalling, on the regulation of the transcriptional activity of Gli1 by SHP. As shown in Figure 5(A), the transcriptional activity of Gli1 was inhibited by Gli3 and SHP. However, co-transfection with both Gli3 and SHP did not result in further suppression compared with transfection of Gli3 or SHP alone. Furthermore, we investigated the recruitment of Gli3 in the promoter region of the *PTCH1* gene, with or without SHP. SHP did not affect the recruitment of Gli3 on the *PTCH1* promoter (Figure 5B).

It was reported that various proteins can regulate Gli activity by affecting the nuclear localization of Gli [41]. In particular, Sufu is a well-known negative regulator of Hedgehog/Gli signalling, which interrupts the nuclear localization of Gli [40]. Therefore we tested the effect of Sufu on the SHP-mediated repression of Gli1 activity. SHP significantly reinforced the

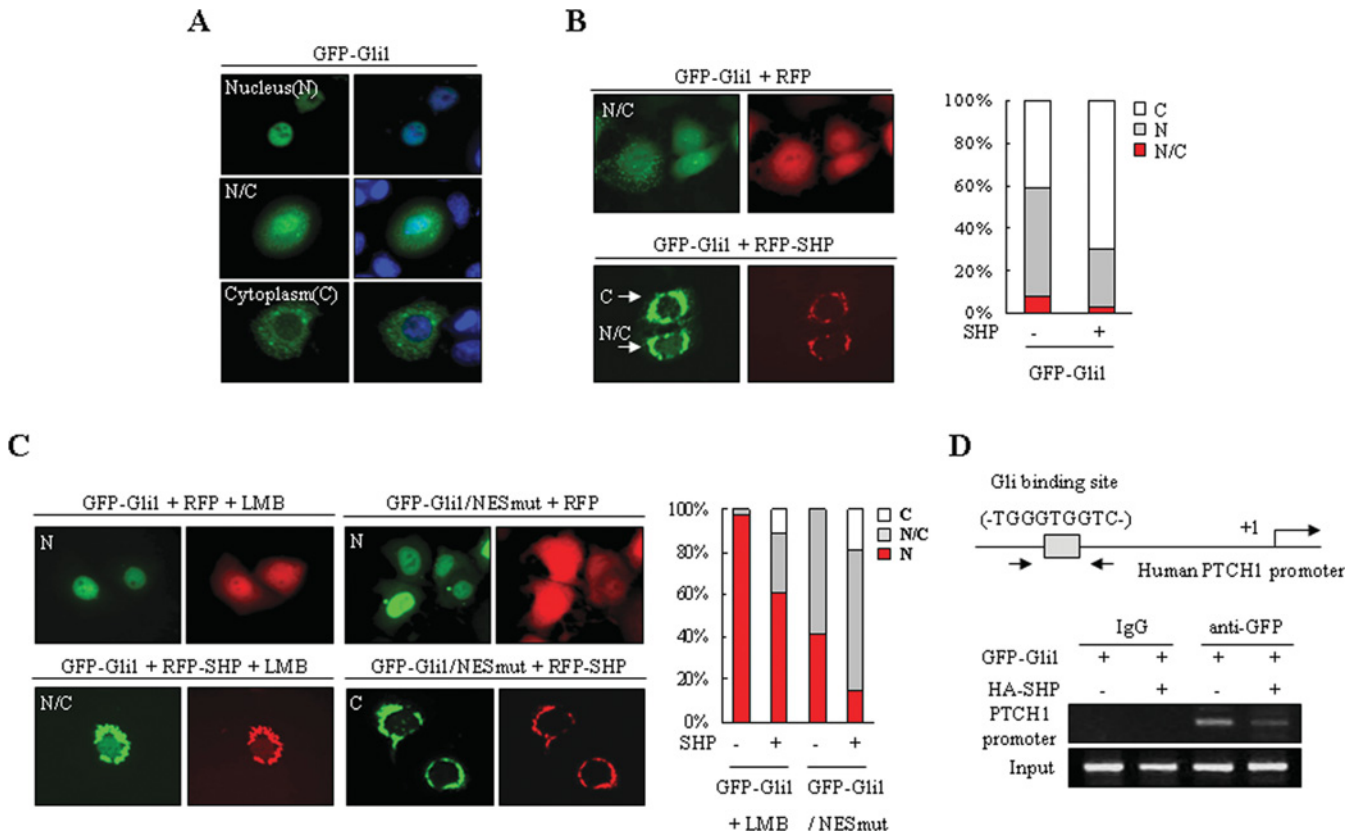

Figure 4 Effect of partial inhibition on Gli1 nuclear localization by SHP

(**A**) The pattern of subcellular localization of GFP–Gli1 in SKBR3 cells. N, nucleus; C, cytoplasm; N/C, nucleus and cytoplasm. (**B**) Subcellular localization of GFP–Gli1 in SKBR3 cells expressing the indicated proteins. Results are representative of three independent experiments. The right-hand panel shows a quantification of the results. Note that the cytosolic GFP–Gli1 is increased in RFP–SHP-transfected cells compared with RFP-transfected cells. (**C**) The effects of LMB and Gli1 NES mutation on SHP-mediated Gli1 subcellular localization. After 24 h of transfection, cells were treated with 5 nM LMB for 8 h. Results shown are representative of three independent experiments. (**D**) The inhibitory effect of SHP on levels of Gli1 binding to the *PTCH1* promoter region (a schematic is shown in the upper panel). After 36 h of transfection with expression plasmids for the indicated proteins, a ChIP assay was performed. Results shown are representative of two independent experiments.

Sufu-mediated repression of Gli1 transcriptional activity in HeLa (Figure 5C) and Huh7 (Supplementary Figure S2A available at http://www.BiochemJ.org/bj/427/bj4270413add.htm) cells. In addition, we identified an interaction between SHP and Gli1 in the presence of Sufu. As shown in Figure 5(D), although the interaction between these proteins was changed only slightly in the presence of Sufu, the interaction between Sufu and Gli1 was significantly enhanced. We further confirmed the effect of Sufu and SHP on the ability of Gli1 to bind to DNA on the *PTCH1* promoter. As shown in Figure 5(E), the result demonstrated that Sufu inhibited the SHP-mediated repression of Gli1 DNA binding. These results suggest that SHP can inhibit the activity of Gli1 by the enhancing the association with Sufu.

SHP-knockdown increases the expression of Gli1 target genes

To confirm the inhibitory effect of SHP overexpression on Gli1 activity, we utilized an SHP-knockdown system using shRNA. A luciferase assay, with the Gli-binding reporter, indicated that Gli1 transcriptional activity was increased in cells co-transfected with SHP shRNA vector compared with control shRNA-expressing cells (Figure 6A, left-hand panel). These results were also observed in a *PTCH1*– and *Bcl-2*–luciferase assay (Figure 6A, right-hand panel). Finally, we investigated the effect of SHP-knockdown on Gli1 target genes. As shown in Figure 6(B), the mRNA expression of Gli1 target genes was increased in cells transfected with the SHP shRNA vector, as determined by RT–PCR (Figure 6B) and qRT-PCR (Figure 6C). We also found that SHP-knockdown resulted in increased expression of *Gli2*. Taken together, these results indicate that SHP modulates the expression of Gli1 target genes by repressing the nuclear localization of Gli1.

DISCUSSION

Increasing evidence indicates that various nuclear receptors are involved in cell proliferation, apoptosis, tumorigenesis and angiogenesis, as well as metabolic disease. In particular, it was reported that many nuclear receptors can repress the Wnt/β-catenin signalling pathway, which plays an important role in the development of various human malignant tumours [31,42]. The activation of the Hedgehog/Gli signalling pathway has also been implicated in the development of cancer in various organs [4,5,7,9,12,32]. Previous reports demonstrated the negative regulatory effects of LXR on Hedgehog signalling in pluripotent mesenchymal cells (marrow stromal cells) and embryonic fibroblasts [34]. However, it was not known whether other nuclear receptors, with anti-cancer potential, repressed the Hedgehog/Gli signalling pathway in cancer cells. In the present study, the effects of various nuclear receptors on the Gli signalling pathway were investigated. We showed that SHP repressed Gli1 transcriptional activity by disturbing Gli1 nuclear localization

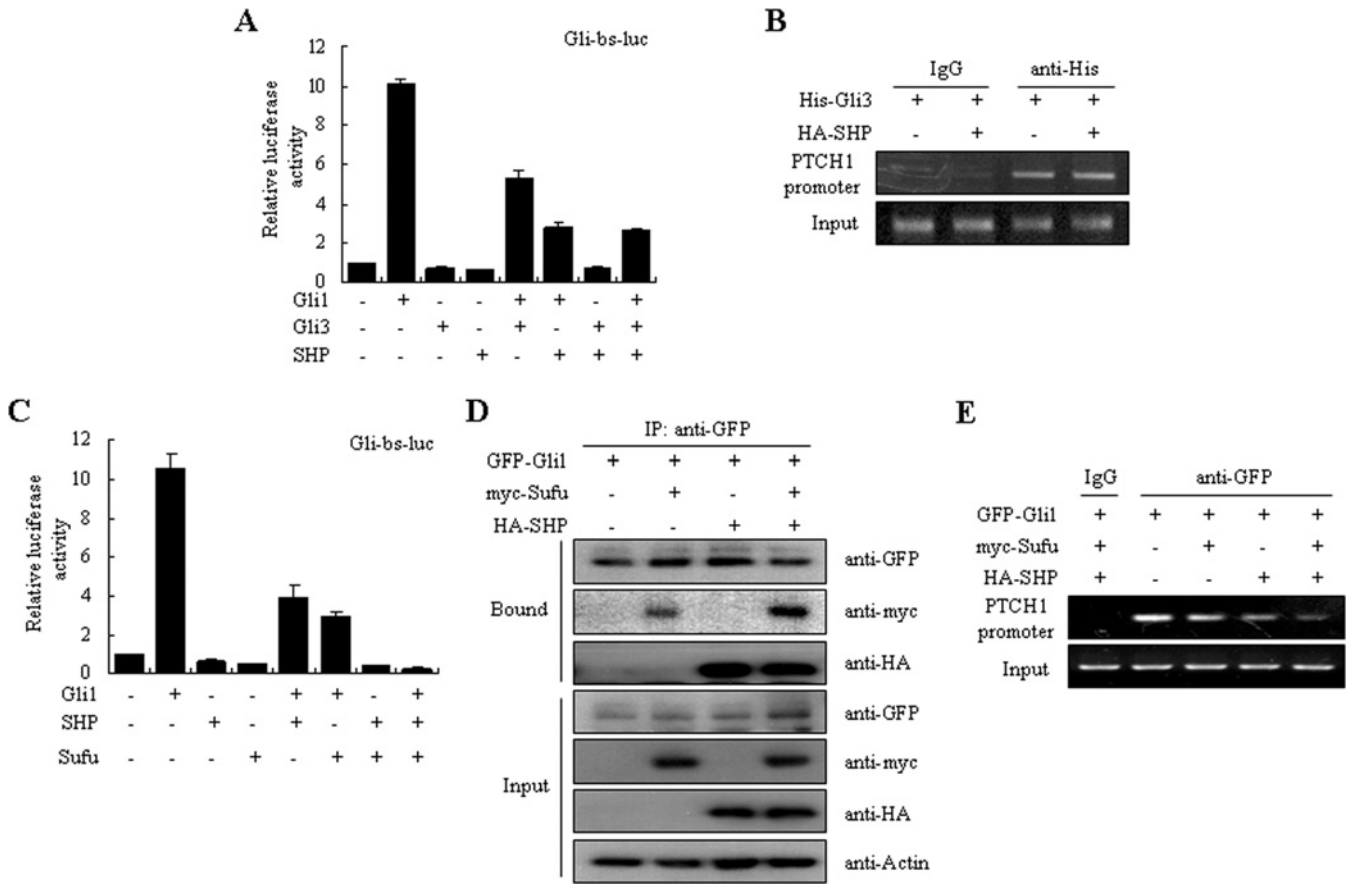

Figure 5 Effects of Gli3 or Sufu on SHP-repressed Gli1 activities

(**A**) Effects of Gli3 on the SHP-mediated inhibition of Gli1 activity. HeLa cells were transfected with Gli1, SHP and Gli3 expression plasmids, along with the Gli-bs-luc reporter plasmid. After 24 h of transfection, cells were harvested and luciferase activity was measured. Results are means ± S.D. ($n = 3$). (**B**) Recruitment of Gli3 by SHP on the *PTCH1* promoter. After 36 h of transfection, a ChIP assay was performed using IgG or the anti-His antibody. (**C**) Effects of Sufu on the SHP-mediated repression of Gli1 activity. HeLa cells were transfected with Gli1, SHP and Sufu expression plasmids, along with the Gli-bs-luc reporter plasmid. After 24 h of transfection, cell lysates were obtained and a luciferase assay was performed. Results are means ± S.D. ($n = 3$). (**D**) Interaction between Sufu and Gli1 in the SHP-overexpressing cells. HeLa cells were transfected with the indicated plasmids. After 36 h of transfection, cell lysates were obtained and immunoprecipitated (IP) with the anti-GFP antibody, followed by Western blotting with the indicated antibodies. (**E**) Effect of Sufu on the SHP-mediated inhibition of Gli1 DNA binding. HeLa cells were transfected with expression plasmids for the indicated proteins. After 36 h of transfection, a ChIP assay was achieved using IgG or anti-GFP antibodies.

via protein–protein interactions, leading to a reduction in the expression of Gli target genes. In addition, the naturally occurring SHP-R34X mutant did not interact with Gli1 and resulted in a loss of the inhibitory potential on Gli1 transcriptional activity (results not shown). Finally, we showed that SHP knockdown may increase the expression of Gli1 target genes due to enhanced Gli1 activity. Thus our findings offer the first evidence that SHP acts as a negative regulator that represses Gli signalling activity.

Numerous studies have suggested that SHP acts as a co-repressor of various nuclear receptors and non-nuclear receptors [23]. In the present study, we identified Gli1 as a protein that interacts with SHP and showed that SHP inhibits the transcriptional activity of Gli1. In general, SHP-mediated transcriptional repression is via recruitment of histone methyltransferase G9a and HDACs. However, G9a and HDACs were not associated with SHP-mediated Gli1 transcriptional suppression. Instead, it is the inhibition of Gli1 nuclear localization which was a mechanism behind this effect. As SHP only partially represses the nuclear localization of Gli, we cannot exclude the possibility that SHP also inhibits Gli1 transcriptional activity in the nucleus via G9a and HDAC-independent mechanisms. Thus additional studies are needed to verify the details of precisely how SHP-mediates repression of Gli transcriptional activity.

Previous studies have suggested that Ras enhances Hedgehog/Gli signalling via increasing Gli1 nuclear localization [43,44]. In the present study, SHP repressed Gli1 nuclear localization, at least in part by protein–protein interactions. Furthermore, we observed that SHP attenuated the K-Ras-enhanced Gli activity and that the naturally occurring SHP-R34X mutant had no effect on this activity (Supplementary Figure 2C). Although the co-operative mechanisms between K-Ras and SHP were not investigated, we consider it possible that collaboration between these factors regulates Gli activity. Further studies are needed to evaluate these and other effects of SHP.

Previous studies have been indicated that SHP plays a role in energy balance in brown fat, glucose homoeostasis, hepatic lipid metabolism and insulin secretion [24–26,45]. Interestingly, two recent studies have suggested that SHP suppresses tumorigenesis by inhibiting cellular proliferation [28,29]. In addition, SHP protected against liver fibrosis by mediating the inhibition of hepatic stellate cells via FXR (farnesoid X receptor) [27]. It has been reported that the Hedgehog/Gli signalling pathway is associated with the development of fibrosis and with energy metabolism, such as in adipogenesis, hepatic lipid metabolism

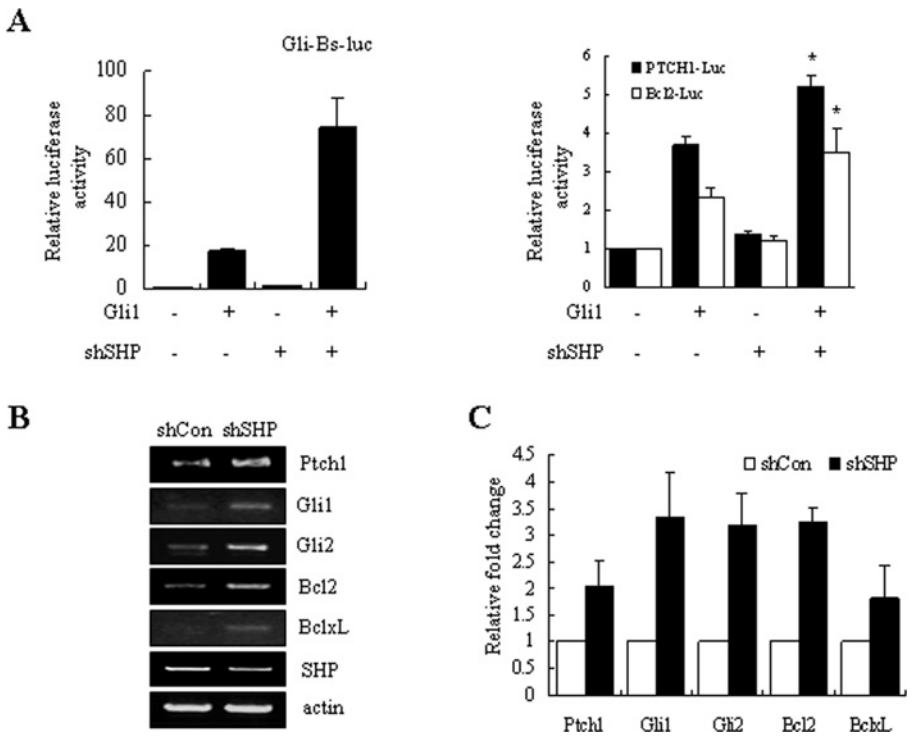

Figure 6 Effect of SHP knockdown on Gli1 transcriptional activity and Gli target gene expression

(**A**) Enhanced Gli1 activity in HeLa cells expressing SHP shRNA. HeLa cells were transfected with expression plasmids for the indicated proteins, along with the Gli-bs-luc (left-hand panel), *PTCH1*–luc and *BCL-2*–luc (right-hand panel) reporter plasmids. After 48 h of transfection, cells were harvested and luciferase activity was determined. Results are means ± S.D. ($n = 3$). *$P < 0.05$ compared with control shRNA-expressing Gli1 transfectants. (**B** and **C**) The increased expression of Hedgehog/Gli target genes upon SHP knockdown. HeLa cells were transfected with shSHP or a control shRNA vector (shCon). After 48 h of transfection, cells were harvested and (**B**) RT–PCR or (**C**) qRT-PCR was performed to assess the level of mRNA. Results from the RT–PCR were quantified using ImageJ version 1.40. Results are representative of two independent experiments.

and insulin secretion, as well as cancer progression [46–48]. In the present study, we found that SHP repressed Gli signalling activity in various cell lines. Taken together with previous findings, our results indicate that it is possible that disruption of the SHP-mediated repression of Gli activity may be involved in the development of fibrosis/cancer and the disruption of energy homoeostasis. It will be very interesting to investigate the role of SHP/Hedgehog/Gli signalling in the development of these pathologies.

In conclusion, our results indicate that SHP is able to repress the transcriptional activity of Gli1, thereby causing a decrease in the expression of Gli target genes. As the loss of SHP is involved in the progression of tumours and metabolic disorders, these findings may provide important information in understanding the role of the Hedgehog/Gli signalling pathway in the development of cancer and metabolic disorder.

AUTHOR CONTRIBUTION

Kyeong Jin Kim and Kook Hwan Kim designed and performed the study. Hyun Kook Cho, Hye Young Kim and Hyeong Hoe Kim provided experimental materials and discussed the study. Kyeong Jin Kim, Kook Hwan Kim and Jaehun Cheong wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

SHP (small heterodimer partner) suppresses the transcriptional activity and nuclear localization of Hedgehog signalling protein Gli1

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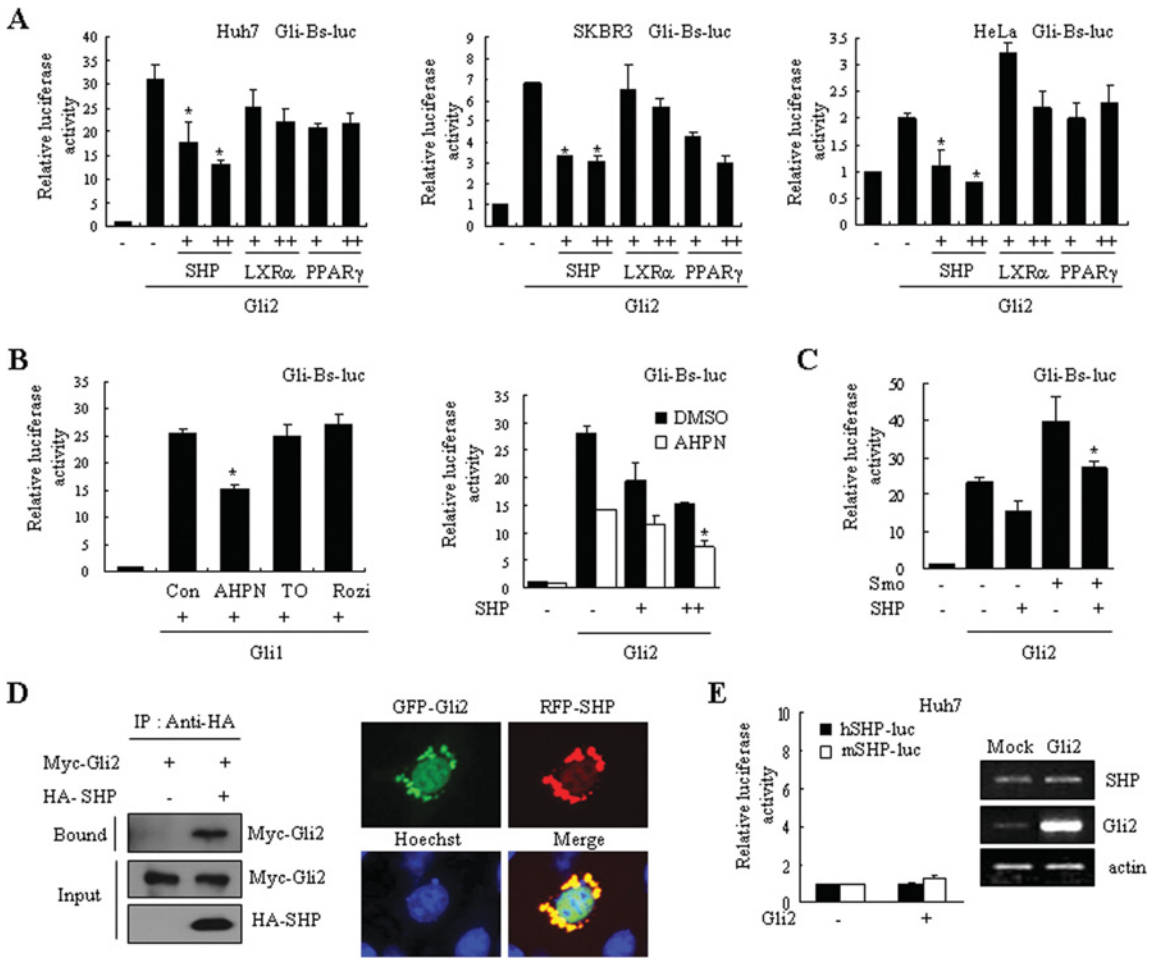

Figure S1 SHP inhibits Gli2 transcriptional activity by protein–protein interaction

(**A**) Identification of SHP as a negative regulator of Gli2 transcriptional activity. Huh7 (left-hand panel), SKBR3 (middle panel) and HeLa (right-hand panel) cells were transfected with 0.1 (+) or 0.2 μg (++) of plasmids expressing the indicated proteins and the Gli-bs-luc reporter plasmid. Relative luciferase activity was then measured. Results are means $\pm$ S.D. ($n=3$). *$P<0.05$ compared with Gli1 transfectants. (**B**) The effect of agonists of various nuclear receptors on Gli2 transcriptional activity (left-hand panel). Huh7 cells were transfected with the 0.1 (+) or 0.2 μg (++) of plasmids expressing the indicated proteins and the Gli-bs-luc reporter plasmid, and treated with the indicated agonists for 24 h as described in Figure 1(B) of the main paper. Cells were harvested and luciferase activity was measured. Results are means $\pm$ S.D. ($n=3$). *$P<0.05$ compared with vehicle-treated Gli2 transfectants. The effect of AHPN on SHP-repressed Gli2 activity (right-hand panel). Huh7 cells were transfected with the plasmid expressing SHP (and the Gli-bs-luc reporter plasmid), and treated with AHPN for 24 h. Cells were harvested and luciferase activity was measured. Results are means $\pm$ S.D. ($n=3$). *$P<0.05$ compared with vehicle-treated transfectants. (**C**) SHP represses the Smo-mediated increase in Gli2 transcriptional activity. Huh7 cells were transfected with the plasmids expressing the indicated proteins and the Gli-bs-luc reporter plasmid, and luciferase activity was measured. Results are the means $\pm$ S.D. ($n=3$). *$P<0.05$ compared with Smo-expressing Gli2 transfectants. (**D**) SHP binds to Gli2. A co-immunoprecipitation (IP) assay (left-hand panel) and fluorescence microscopy analysis (right-hand panel) were performed. Results shown are representative of two or three independent experiments. (**E**) Gli2 has no effect on *SHP* gene expression. Huh7 cells were transfected with plasmid expressing Gli2 and the indicated reporter constructs. Luciferase activity was measured (left-hand panel) and RT–PCR was performed (right-hand panel). Luciferase results are the means $\pm$ S.D. ($n=3$).

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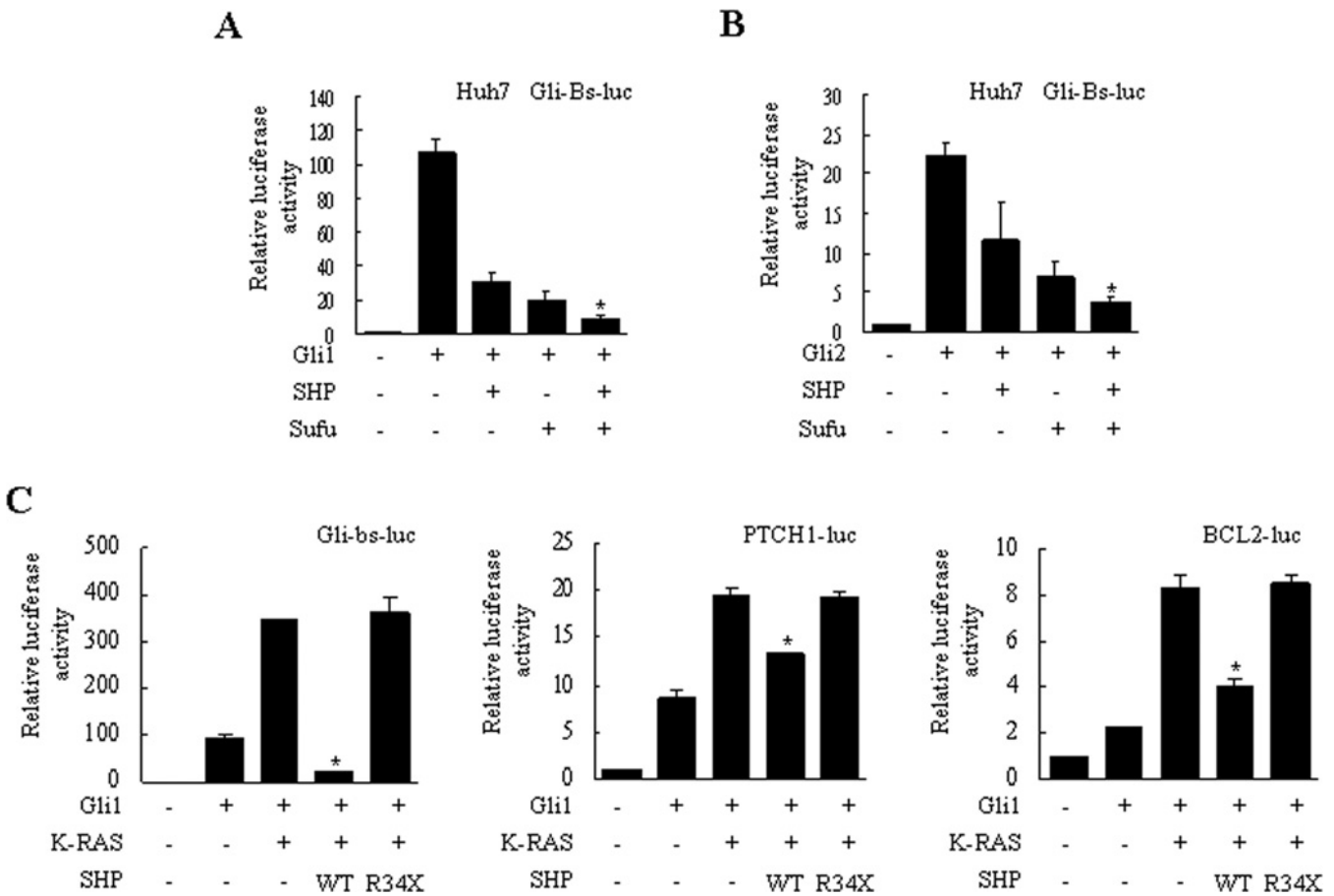

Figure S2 Effect of SHP on Sufu-repressed Gli activity and K-Ras-enhanced Gli activity

(**A** and **B**) Huh7 cells were transfected with the plasmids expressing the indicated proteins and the Gli-bs-luc reporter plasmid, and luciferase activity was measured. Results are means ± S.D. ($n = 3$). *$P < 0.05$ compared with Sufu-expressing Gli transfectants. (**C**) The plasmids expressing the indicated proteins and luciferase reporter constructs were transfected into Huh7 cells and luciferase activity was measured. Results are means ± S.D. ($n = 3$). *$P < 0.05$ compared with K-Ras-expressing Gli1 transfectants. WT, wild-type; R34X, SHP-R34X mutant.

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